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| (54) Title: <b>SPECIFIC BINDING MEMBERS FOR HUMAN TRANSFORMING GROWTH FACTOR BETA; MATERIALS AND METHODS</b>   |  |  |  |
| (57) Abstract<br><p>Specific binding members comprising human antibody antigen binding domains specific for human transforming growth factor beta (TGF<math>\beta</math>) bind specifically isoforms TGF<math>\beta</math>2 and TGF<math>\beta</math>1 or both, preferentially compared with TGF<math>\beta</math>3. Specific binding members may be isolated and utilised in the treatment of disease, particularly fibrotic disease and also immune/inflammatory diseases. Therapeutic utility is demonstrated using <i>in vitro</i> and <i>in vivo</i> models. Full sequence and binding information is provided, including epitope sequence information for a particularly advantageous specific binding member which binds the active form of TGF<math>\beta</math>2, neutralising its activity, but does not bind the latent form.</p>   |  |  |  |

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SPECIFIC BINDING MEMBERS FOR HUMAN TRANSFORMING  
GROWTH FACTOR BETA; MATERIALS AND METHODS

This invention relates to specific binding members for human transforming growth factor beta (TGF $\beta$ ) and materials and methods relating thereto. In particular, it relates to specific binding members comprising antibody binding domains; for example, human antibodies. Human antibodies against human TGF $\beta$  may be isolated and utilised in the treatment of disease, particularly fibrotic disease and also immune/inflammatory diseases. The isolation of antiself antibodies from antibody segment repertoires displayed on phage has been described (A.D.Griffiths et al. EMBO J. 12, 725-734, 1993; A. Nissim et al. EMBO J. 13, 692-698, 1994; A.D. Griffiths et al. 13, 3245-3260, 1994; C.Barbas et al. Proc. Natl. Acad. Sci. USA 90, 10003-10007 1993; WO93/11236). However, the present invention provides specific antibodies against a particular isoforms of TGF $\beta$ , which antibodies have unexpected and advantageous properties.

TGF $\beta$  is a cytokine known to be involved in many cellular processes such as cell proliferation and differentiation, embryonic development, extracellular matrix formation, bone development, wound healing, hematopoiesis and immune and inflammatory responses (A.B. Roberts & M. Sporn 1990 pp419-472 in Handbook of Experimental Pharmacology eds M.B. Sporn &

A.B. Roberts, Springer Heidelberg; J.Massague et al. Annual Rev. Cell Biol. 6, 597-646, 1990).

The accumulation of excessive extra-cellular matrix is associated with various fibrotic diseases.

5 Thus there is a need to control agents such as TGF $\beta$ 1 and TGF $\beta$ 2 to prevent their deleterious effects in such diseases and this is one application of human antibodies to human TGF $\beta$ .

The modulation of immune and inflammatory  
10 responses by TGFbetas includes (i) inhibition of proliferation of all T-cell subsets (ii) inhibitory effects on proliferation and function of B lymphocytes (iii) down-regulation of natural-killer cell activity and the T-cell response (iv) regulation of cytokine  
15 production by immune cells (v) regulation of macrophage function and (vi) leucocyte recruitment and activation.

A further application of antibodies to TGF $\beta$  may be in the treatment of immune/inflammatory diseases  
20 such as rheumatoid arthritis, where these functions need to be controlled.

It is a demanding task to isolate an antibody fragment specific for TGF $\beta$  of the same species. Animals do not normally produce antibodies to self  
25 antigens, a phenomenon called tolerance (G.J. Nossal Science 245, 147-153, 1989). In general, vaccination with a self antigen does not result in production of circulating antibodies. It is therefore difficult to



raise human antibodies to human self antigens. There are also in addition, ethical problems in vaccinating humans. In relation to the raising of non-human antibodies specific for TGF $\beta$ , there are a number of problems. TGF $\beta$  is an immunosuppressive molecule and further, there is strong conservation of sequence between human and mouse TGF $\beta$  molecules. Mouse and human TGF $\beta$ 1 only differ by one amino acid residue, an alanine (human) to serine (mouse) change at a buried residue (R.Derynck et al. J.Biol. Chem. 261, 4377-4379, 1986). Mouse and human TGF $\beta$ 2 only differ at three residues; residue 59 (T mouse, S human); residue 60 (K mouse, R human) and residue 94 (N mouse; K human). This makes it difficult to raise antibodies in mice against human TGF $\beta$ . Further, any antibodies raised may only be directed against a restricted set of epitopes.

Polyclonal antibodies binding to human TGF $\beta$ 1 and human TGF $\beta$ 2 against both neutralising and non-neutralising epitopes have been raised in rabbit (Danielpour et al. Growth Factors 2 61-71, 1989; A. Roberts et al. Growth Factors 3, 277-286, 1990), chicken (R&D Systems, Minneapolis) and turkey (Danielpour et al. J. Cell Physiol. 138, 79-86, 1989). Peptides representing partial TGF $\beta$  sequences have also been used as immunogens to raise neutralising polyclonal antisera in rabbits (W.A Border et al. Nature 346, 371-374, 1990; K.C. Flanders Biochemistry

27, 739-746, 1988; K.C. Flanders et al, Growth Factors  
3, 45-52, 1990). In addition there have been limited  
reports of isolation of mouse monoclonals against  
TGF $\beta$ . Following immunisation with bovine TGF $\beta$ 2  
5 (identical to human TGF $\beta$ 2), three non-neutralising  
monoclonal antibodies were isolated that are specific  
for TGF $\beta$ 2 and one neutralising antibody that is  
specific for TGF $\beta$ 1 and TGF $\beta$ 2 (J.R. Dasch et al. J.  
Immunol. 142, 1536-1541, 1989). In another report,  
10 following immunisation with human TGF $\beta$ 1, neutralising  
antibodies were isolated which were either specific  
for TGF $\beta$ 1 or cross-reacted with TGF $\beta$ 1, TGF $\beta$ 2 and  
TGF $\beta$ 3 (C. Lucas et al. J.Immunol. 145, 1415-1422,  
1990). A neutralising mouse monoclonal antibody which  
15 binds both TGF $\beta$ 2 and TGF $\beta$ 3 isoforms is available  
commercially from Genzyme Diagnostics.

The present text discloses the first isolation of  
human antibodies directed against human TGF $\beta$ 1 and  
against human TGF $\beta$ 2. A mouse monoclonal antibody  
20 directed against human TGF $\beta$ 1 is available from R&D  
Systems. This antibody only weakly neutralises TGF $\beta$ 1  
in a neutralisation assay. Neutralising mouse  
monoclonal antibodies have also been generated from  
mice immunised with human TGF $\beta$ 1 peptides comprising  
25 amino acid positions 48 to 60 (antibody reactive with  
TGF $\beta$ 1, TGF $\beta$ 2 and TGF $\beta$ 3) and amino acid positions 86-101  
(antibody specific for TGF $\beta$ 1; M. Hoefer & F.A. Anderer  
Cancer Immunol. Immunother. 41, 302-308, 1995).

Phage antibody technology (WO92/01047; PCT/GB92/00883; PCT/GB92/01755; WO93/11236) offers the ability to isolate directly human antibodies against human TGF $\beta$ . In application WO93/11236 the isolation  
5 of antiseif antibodies from phage display libraries was disclosed and it was suggested that antibodies specific for TGF $\beta$  could be isolated from phage display libraries.

The present application shows that antibodies of  
10 differing specificities for TGF $\beta$  molecules may be isolated. TGF $\beta$ 1, TGF $\beta$ 2 and TGF $\beta$ 3 are a closely related group of cytokines. They are dimers consisting of two 112 amino acid monomers joined by an interchain disulphide bridge. TGF $\beta$ 1 differs from  
15 TGF $\beta$ 2 by 27 mainly conservative changes and from TGF $\beta$ 3 by 22 mainly conservative changes. These differences have been related to the 3D structure (M.Schlunegger & M.G.Grutter Nature 358, 430-434, 1992). The present applicants have isolated antibodies which are  
20 essentially specific for TGF $\beta$ 1 (very low cross-reactivity with TGF $\beta$ 2); antibodies which are essentially specific for TGF $\beta$ 2 (very low cross-reactivity TGF $\beta$ 1); and antibodies which bind both TGF $\beta$ 1 and TGF $\beta$ 2. Hence, these three different types  
25 of antibodies, each type with distinctive binding specificities must recognise different epitopes on the TGF $\beta$  molecules. These antibodies have low cross-reactivity with TGF $\beta$ 3 as assessed by binding studies

using biosensor assays (e.g. BIAcore™), ELISA and radioreceptor assays. The most extensively studied antibody, 6B1 IgG4, shows 9% cross-reactivity with TGFβ3 as compared with TGFβ2, as determined by their relative dissociation constants, determined using a biosensor.

TGFβ isoforms are initially exported from cells as inactive, latent forms (R. Pircher et al, Biochem. Biophys. Res. Commun. 136, 30-37, 1986; L.M. Wakefield et al., *Growth Factors* 1, 203-218, 1989). These inactive forms are activated by proteases in plasma to generate the active form of TGFβ. It is this active form of TGFβ2 which binds to receptors promoting the deposition of extracellular matrix and the other biological effects of TGFβ. The active form of TGFβ represents a relatively low proportion of TGFβ that is in the plasma. Therefore, for a neutralising antibody against TGFβ to be most effective at preventing fibrosis the antibody should recognise the active but not the latent form. In Example 6, it is demonstrated that a preferred antibody of this invention ("6B1 IgG4") recognises the active but not the latent form of TGFβ2.

The epitope of 6B1 IgG4 has been identified using a combination of peptide display libraries and inhibition studies using peptides from the region of TGFβ2 identified from phage selected from the peptide phage display library. This is described in Examples

11 and 14. The sequence identified from the peptide library is RVL<sub>56</sub>SL and represents amino acids 60 to 64 of TGF $\beta$ 2 (Example 11). The antibody 6B1 IgG4 has also been shown to bind to a peptide corresponding to amino acids 56 to 69 of TGF $\beta$ 2 (TQHSRVL<sub>56</sub>SLYNTIN) with a three amino acid (CGG) extension at the N-terminus. RVL<sub>56</sub>SL is the minimum epitope, 6B1 IgG4 is likely to bind to further adjacent amino acids. Indeed, if the epitope is three dimensional there may be other non-contiguous sequences to which the antibody will bind. 6B1 IgG4 shows much weaker binding to the peptide corresponding to amino acids 56 to 69 of TGF $\beta$ 1 (CGG-TQYSKVLSLYNQHN).

The results of Example 14 support the assignment of the epitope of 6B1 IgG4 on TGF $\beta$ 2 to the amino acids in the region of residues 60 to 64. The peptide used in this example, residues 56 to 69, corresponds to the amino acids of alpha helix H3. (M.P. Schlunegger & M.G. Grutter Nature 358 430-434, 1992; also known as the  $\alpha$ 3 helix (S. Daopin et al Proteins: Structure, Function and Genetics 17 176-192, 1993). TGF $\beta$ 2 forms a head-to-tail dimer with the alpha helix H3 (also referred to as the heel) of one subunit forming an interface with finger regions (including residues 24 to 37 and residues in the region of amino acids 91 to 95; also referred to as fingers 1 and 2) from the other subunit (S. Daopin et al supra). It has been proposed that the primary structural features which interact with the TGF $\beta$ 2 receptor consist of amino acids at the C-

terminal end of the alpha helix H3 from one chain together with residues of fingers 1 and 2 of the other chain (D.L. Griffith et al Proc. Natl. Acad. Sci. USA 93 878-883,, 1996). The identification of an epitope  
5 for 6B1 IgG4 within the alpha helix H3 of TGF $\beta$ 2 is consistent with 6B1 IgG4 preventing receptor binding and neutralising the biological activity of TGF $\beta$ 2.

As noted above if the epitope for 6B1 IgG4 is three dimensional there may be other non-contiguous  
10 amino acids to which the antibody may bind.

There is earlier advice that antibodies directed against this region of TGF $\beta$ 2 may be specific for TGF $\beta$ 2 and neutralise its activity. Flanders et al (Development 113 183-191, 1991) showed that polyclonal  
15 antisera could be raised in rabbits against residues 50 to 75 of mature TGF $\beta$ 2 and that these antibodies recognised TGF $\beta$ 2 but the TGF $\beta$ 1 in Western blots. In an earlier paper, K.C. Flanders et al (Biochemistry 27 739-746, 1988) showed that polyclonal antisera raised  
20 in rabbits against amino acids 50 to 75 of TGF $\beta$ 1 could neutralise the biological activity of TGF $\beta$ 1. The antibody isolated in this application 6B1 IgG4 is a human antibody directed against the amino acids in this region which neutralises the biological activity  
25 of human TGF $\beta$ 2. It is surprising that such a neutralising antibody against TGF $\beta$ 2 can be isolated in humans (where immunisation with a peptide cannot be used for ethical reasons) directly from a phage

display antibody repertoire.

The knowledge that the residues of the alpha helix H3 form a neutralising epitope for TGF $\beta$ 2 means that phage displaying neutralising antibodies are

5 obtainable by selection from phage antibody repertoires by binding to a peptide from this region coupled to a carrier protein such as bovine serum albumin or keyhole limpet haemocyanin. This approach may be applied to select antibodies which are capable

10 of neutralising the biological activity of TGF $\beta$ 1 by selecting on the peptide TQYSKVLSTLYNQHN coupled to a carrier protein. It is possible that such an approach may be extended to peptides from receptor binding regions of TGF $\beta$  isoforms, other than the H3 alpha

15 helix.

It has further been demonstrated by the present inventors that antibodies specific for TGF $\beta$  are obtainable by isolation from libraries derived from different sources of immunoglobulin genes: from

20 repertoires of natural immunoglobulin variable domains, e.g. from immunised or non-immunised hosts; and synthetic repertoires derived from germline V genes combined with synthetic CDR3s. The properties of these antibodies in single chain Fv and whole IgG4

25 format are described.

As noted above WO93/11236 suggested that human antibodies directed against human TGF $\beta$  could be isolated from phage display libraries. Herein it is

shown that the phage display libraries from which  
antibodies were isolated in WO93/11236 may be  
utilised as a source of human antibodies specific for  
particular human TGF $\beta$  isoforms. For instance, in  
5 example 1 of the present application, the antibody 1A-  
E5 specific for TGF $\beta$ 1 and the antibodies 2A-H11 and  
2A-A9 specific for TGF $\beta$ 2 were isolated from the  
"synthetic library" described in examples 5 to 7 of  
WO93/11236 and in Nissim et al. (1994; supra). Also,  
10 the phage display library derived from peripheral  
blood lymphocytes (PBLs) of an unimmunised human  
(examples 1 to 3 of WO93/11236) was the source for the  
antibody 1B2 specific for TGF $\beta$ 1. Phage display  
libraries made subsequently utilising antibody genes  
15 derived from human tonsils and bone marrow, have also  
provided sources of antibodies specific for human  
TGF $\beta$ . Thus human TGF $\beta$  is an example of a human self  
antigen to which antibodies may be isolated from  
"large universal libraries". Human antibodies against  
20 human TGF $\beta$  with improved properties can be obtained by  
chain shuffling for instance combining the VH domains  
of antibodies derived from one library with the VL  
domains of another library thus expanding the pool of  
VL partners tested for each VH domain. For instance,  
25 the antibodies 6B1, 6A5 and 6H1 specific for TGF $\beta$ 2  
utilise the 2A-H11 VH domain isolated from the  
"synthetic library" combined with a light chain from  
the PBL library.



Thus the VH and VL domains of antibodies specific for TGF $\beta$  can be contributed from phage display libraries derived from rearranged V genes such as those in PBLs, tonsil and bone marrow and from V domains derived from cloned germline V segments combined with synthetic CDRs. There are also shown to be a diverse range of antibodies which are specific for TGF $\beta$ 1 or TGF $\beta$ 2. The antibodies which have been isolated both against TGF $\beta$ 1 and TGF $\beta$ 2 have mainly utilised V genes derived from VH germlines of the VH3 family. A wider variety of light chain variable regions have been used, of both the lambda and kappa types.

Individual antibodies which have been isolated have unexpectedly advantageous properties. For example, the antibodies directed against TGF $\beta$ 2 (6H1, 6A5 and 6B1) have been shown to bind to TGF $\beta$ 2 with slow off-rates (off-rate constants  $k_{\text{off}}$  of the order of  $10^{-3} \text{ s}^{-1}$  and dissociation constants of less than  $10^{-6} \text{ M}$ ) to neutralise TGF $\beta$ 2 activity in in vitro assays and to be potent in in vivo applications. The antibody 6B1 IgG4 has been shown to bind specifically to TGF $\beta$ 2 in immunohistochemistry in mammalian tissues and not to cross-react with other antigens in human tissues. The properties of these antibodies may make them particularly suitable for therapeutic applications. The fact that these antibodies share the same heavy chain, shows that VH domains can be effective with a

number of different light chains, although there may be differences in potency or subtle changes of epitope with different light chains. As shown in Examples 3 and 4 and Tables 4 and 5, 6B1 IgG4 is the most potent antibody in neutralising TGF $\beta$ 2 activity in the radioreceptor assay and the TF1 proliferation assay. Its properties may however be expected to be qualitatively similar to the antibodies 6A5 and 6H1 with which it shares a common VH domain. Thus the reduction in neural scarring observed on treatment with 6A5 single chain Fv and 6H1 IgG4 shown in Example 5 would be expected to be reproduced with 6B1. The antibodies directed against TGF $\beta$ 1 (particularly 1B2 and its derivatives) also have unexpectedly advantageous properties. Antibody 27C1/10A6 derived from 1B2 by chain shuffling, spiking and conversion into whole antibody IgG4, has been shown to be potent in an *in vitro* scarring model. The VH domain of this antibody was derived by site directed "spiking" mutagenesis from the parent antibody 7A3. A large number of spiked clones were obtained which show similar properties in *in vitro* assays. There can be a number of changes in CDR3 of the VH compared to 27C1, for instance, 28A-H11 differs in 7 of the 14 positions, 2 of which are non-conservative changes. Thus there may be up to 50% of the residues in the VH CDR3 changed without affecting binding properties.

Antibodies specific for human TGF $\beta$ 1 and human

TGF $\beta$ 2 have been shown to be effective in animal models for the treatment of fibrotic diseases and other diseases such as rheumatoid arthritis where TGF $\beta$  is overexpressed. Antibodies against TGF $\beta$  have been

5 shown to be effective in the treatment of glomerulonephritis (W.A Border et al. Nature 346, 371-374, 1990); neural scarring (A. Logan et al. Eur. J. Neurosci. 6, 355-363, 1994); dermal scarring (M. Shah et al. Lancet 339, 213-214 1992; M. Shah et al. J. Cell

10 Science 107, 1137-1157, 1994; M. Shah et al. 108, 985-1002, 1995); lung fibrosis (S.N. Giri et al. Thorax 48, 959-966, 1993); arterial injury (Y.G. Wolf, L.M. Rasmussen & E. Ruoslahti J. Clin. Invest. 93, 1172-1178, 1994) and rheumatoid arthritis (Wahl et al J.

15 Exp. Medicine 177, 225-230, 1993). It has been suggested that TGF $\beta$ 3 acts antagonistically to TGF $\beta$ 1 and TGF $\beta$ 2 in dermal scarring (M. Shah et al. 1995 supra.). Therefore, antibodies to TGF $\beta$ 1 or TGF $\beta$ 2 with apparent low cross-reactivity to TGF $\beta$ 3, as assessed by

20 binding studies using a biosensor assay (e.g. BIAcore™), ELISA or a radioreceptor assay, as disclosed in this application, that is to say antibodies which bind preferentially to TGF $\beta$ 1 or TGF $\beta$ 2 compared with TGF $\beta$ 3, should be advantageous in this

25 and other conditions such as fibrotic conditions in which it is desirable to counteract the fibrosis promoting effects of TGF $\beta$ 1 and TGF $\beta$ 2. An antibody which cross-reacts strongly with TGF $\beta$ 3 has however had

an effect in an animal model of rheumatoid arthritis (Wahl et al., 1993, *supra*).

There are likely to be applications further to the above mentioned conditions, as there are several other *in vitro* models of disease where antibodies against TGF $\beta$  have shown promise of therapeutic efficacy. Of particular importance may be the use of antibodies against TGF $\beta$  for the treatment of eye diseases involving ocular fibrosis, including proliferative retinopathy (R.A. Pena et al. (ref. below) , retinal detachment and post glaucoma (P.T. Khaw et al., *Eye* 8 188-195, 1994) drainage surgery. Connor et al. (*J. Clin. Invest* 83 1661-1666, 1989) showed that much higher levels of TGF $\beta$ 2 were present in vitreous aspirates from patients with intraocular fibrosis associated with proliferative retinopathy compared with patients with uncomplicated retinal detachment without ocular fibrosis and that the biological activity of this TGF $\beta$ 2 could be neutralised with antibodies directed against TGF $\beta$ 2. Moreover, Pena et al. (*Invest. Ophthalmology. Vis. Sci.* 35: 2804-2808, 1994) showed that antibodies against TGF $\beta$ 2 inhibit collagen contraction stimulated by TGF $\beta$ 2. Contraction of the vitreous gel by fibroblasts and other cell types plays a critical role in the proliferative retinopathy disease process, a process thought to be mediated by TGF $\beta$ 2.

There is other evidence pointing to TGF $\beta$ 2 being

the most important TGF $\beta$  isoform promoting intraocular fibrosis. TGF $\beta$ 2 has been shown to be the predominant isoform of TGF $\beta$  in the neural retina, retinal pigment epithelium-choroid and vitreous of the human eye

5 (Pfeffer et al. *Exp. Eye Res.* 59: 323-333, 1994) and found in human aqueous humour in specimens from eyes undergoing cataract extraction with intraocular lens implantation (Jampel et al. *Current Eye Research* 9: 963-969, 1990). Non-transformed human retinal pigment  
10 epithelial cells predominantly secrete TGF $\beta$ 2 (Kvanta *Ophthalmic Res.* 26: 361-367, 1994).

Other diseases which have potential for treatment with antibodies against TGF $\beta$  include adult respiratory distress syndrome, cirrhosis of the liver, post  
15 myocardial infarction, post angioplasty restenosis, keloid scars and scleroderma. The increase level of expression of TGF $\beta$ 2 in osteoporosis (Erlenbacher et al. *J. Cell Biol.* 132: 195-210, 1996) means that this is a disease potentially treatable by antibodies  
20 directed against TGF $\beta$ 2.

The use of antibodies against TGF $\beta$  for the treatment of diseases has been the subject of patent applications for fibrotic disease (WO91/04748); dermal scarring (WO92/17206); macrophage deficiency diseases  
25 (PCT/US93/00998); macrophage pathogen infections (PCT/US93/02017); neural scarring (PCT/US93/03068); vascular disorders (PCT/US93/03795); prevention of cataract (WO95/13827). The human antibodies against

human TGF $\beta$  disclosed in this application should be valuable in these conditions.

It is shown herein that the human antibodies both against human TGF $\beta$ 1 and against human TGF $\beta$ 2 can be effective in the treatment of fibrosis in animal models of neural scarring and glomerulonephritis in either single chain Fv and whole antibody format. This is the first disclosure of the effectiveness of antibodies directed only against TGF $\beta$ 2 as sole treatment in these indications, although some effectiveness of antibodies against TGF $\beta$ 2 only has been observed in a lung fibrosis model (Giri et al. Thorax 48, 959-966, 1993 supra). The effectiveness of the human antibodies against human TGF $\beta$  in treatment of fibrotic disease has been determined by measuring a decrease in the accumulation of components of the extracellular matrix, including fibronectin and laminin in animal models.

The evidence of efficacy of the antibodies against TGF $\beta$ 2 and TGF $\beta$ 1 describe herein in prevention of neural scarring in the animal model experiment means that these antibodies are likely to be effective in other disease states mediated by TGF $\beta$ . For comparison, antisera isolated from turkeys directed against TGF $\beta$  isoforms by Danielpour et al. (Cell Physiol. 138: 79-86, 1989) have been shown to be effective in the prevention of dermal scarring (Shah et al. J. Cell Science 108: 985-1002, 1995), neural

scarring (Logan et al., *supra*) and in *in vitro* experiments relating to proliferative retinopathy (Connor et al., *supra*).

## 5    *TERMINOLOGY*

### *Specific binding member*

This describes a member of a pair of molecules which have binding specificity for one another. The members of a specific binding pair may be naturally derived or wholly or partially synthetically produced. One member of the pair of molecules has an area on its surface, or a cavity, which specifically binds to and is therefore complementary to a particular spatial and polar organisation of the other member of the pair of molecules. Thus the members of the pair have the property of binding specifically to each other. Examples of types of specific binding pairs are antigen-antibody, biotin-avidin, hormone-hormone receptor, receptor-ligand, enzyme-substrate. This application is concerned with antigen-antibody type reactions.

### *Antibody*

25        This describes an immunoglobulin whether natural or partly or wholly synthetically produced. The term also covers any polypeptide or protein having a binding domain which is, or is homologous to, an

antibody binding domain. These can be derived from natural sources, or they may be partly or wholly synthetically produced. Examples of antibodies are the immunoglobulin isotypes and their isotypic subclasses; fragments which comprise an antigen binding domain such as Fab, scFv, Fv, dAb, Fd; and diabodies.

It is possible to take monoclonal and other antibodies and use techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP-A-184187, GB 2188638A or EP-A-239400. A hybridoma or other cell producing an antibody may be subject to genetic mutation or other changes, which may or may not alter the binding specificity of antibodies produced.

As antibodies can be modified in a number of ways, the term "antibody" should be construed as covering any specific binding member or substance having a binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an



immunoglobulin binding domain, whether natural or wholly or partially synthetic. Chimeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore  
5 included. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023.

It has been shown that fragments of a whole antibody can perform the function of binding antigens.  
10 Examples of binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S.  
15 et al., Nature 341, 544-546 (1989)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')<sub>2</sub> fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a  
20 peptide linker which allows the two domains to associate to form an antigen binding site (Bird et al, Science, 242, 423-426, 1988; Huston et al, PNAS USA, 85, 5879-5883, 1988); (viii) bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies",  
25 multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al Proc. Natl. Acad. Sci. USA 90 6444-6448, 1993).

Diabodies are multimers of polypeptides, each

polypeptide comprising a first domain comprising a binding region of an immunoglobulin light chain and a second domain comprising a binding region of an immunoglobulin heavy chain, the two domains being  
5 linked (e.g. by a peptide linker) but unable to associate with each other to form an antigen binding site: antigen binding sites are formed by the association of the first domain of one polypeptide within the multimer with the second domain of another  
10 polypeptide within the multimer (WO94/13804).

Where bispecific antibodies are to be used, these may be conventional bispecific antibodies, which can be manufactured in a variety of ways (Holliger, P. and Winter G. Current Opinion Biotechnol. 4, 446-449  
15 (1993)), eg prepared chemically or from hybrid hybridomas, or may be any of the bispecific antibody fragments mentioned above. It may be preferable to use scFv dimers or diabodies rather than whole antibodies. Diabodies and scFv can be constructed  
20 without an Fc region, using only variable domains, potentially reducing the effects of anti-idiotypic reaction. Other forms of bispecific antibodies include the single chain "Janusins" described in Traunecker et al, Embo Journal, 10, 3655-3659, (1991).

25 Bispecific diabodies, as opposed to bispecific whole antibodies, may also be particularly useful because they can be readily constructed and expressed in *E.coli*. Diabodies (and many other polypeptides

such as antibody fragments) of appropriate binding specificities can be readily selected using phage display (WO94/13804) from libraries. If one arm of the diabody is to be kept constant, for instance, with  
5 a specificity directed against antigen X, then a library can be made where the other arm is varied and an antibody of appropriate specificity selected.

#### *Antigen binding domain*

10 This describes the part of an antibody which comprises the area which specifically binds to and is complementary to part or all of an antigen. Where an antigen is large, an antibody may only bind to a particular part of the antigen, which part is termed  
15 an epitope. An antigen binding domain may be provided by one or more antibody variable domains. Preferably, an antigen binding domain comprises an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH).

20

#### *Specific*

This may be used to refer to the situation in which one member of a specific binding pair will not show any significant binding to molecules other than  
25 its specific binding partner(s). The term is also applicable where e.g. an antigen binding domain is specific for a particular epitope which is carried by a number of antigens, in which case the specific

binding member carrying the antigen binding domain will be able to bind to the various antigens carrying the epitope.

5    *Neutralisation*

This refers to the situation in which the binding of a molecule to another molecule results in the abrogation or inhibition of the biological effector function of the another molecule.

10

*Functionally equivalent variant form*

This refers to a molecule (the variant) which although having structural differences to another molecule (the parent) retains some significant  
15    homology and also at least some of the biological function of the parent molecule, e.g. the ability to bind a particular antigen or epitope. Variants may be in the form of fragments, derivatives or mutants. A variant, derivative or mutant may be obtained by  
20    modification of the parent molecule by the addition, deletion, substitution or insertion of one or more amino acids, or by the linkage of another molecule. These changes may be made at the nucleotide or protein level. For example, the encoded polypeptide may be a  
25    Fab fragment which is then linked to an Fc tail from another source. Alternatively, a marker such as an enzyme, flourescein, etc, may be linked.

*Comprise*

This is generally used in the sense of include, that is to say permitting the presence of one or more features or components.

5

The present invention generally provides a specific binding member comprising an antibody antigen binding domain. More particularly it provides a specific binding member for TGF $\beta$ , particularly the  
10 isoforms TGF $\beta$ 2, TGF $\beta$ 1, or TGF $\beta$ 1 and TGF $\beta$ 2.

The present invention provides a specific binding member which comprises a human antibody antigen binding domain specific for TGF $\beta$ 1 and/or TGF $\beta$ 2 and which has low cross reactivity with TGF $\beta$ 3. The cross-  
15 reactivity may be as assessed using any or all of the following assays: biosensor (e.g. BIAcore™), ELISA and radioreceptor. The present invention provides specific binding member which comprises a human antibody antigen binding domain specific for TGF $\beta$ 1  
20 and/or TGF $\beta$ 2 which binds preferentially to these isoforms compared with TGF $\beta$ 3.

The TGF $\beta$  may be human TGF $\beta$ .

The specific binding member may be in the form of an antibody fragment such as single chain Fv (scFv).  
25 Other types of antibody fragments may also be utilised such as Fab, Fab', F(ab')<sub>2</sub>, Fabc, Facb or a diabody (G.Winter & C.Milstein Nature 349, 293-299, 1991; WO94/13804). The specific binding member may be in

the form of a whole antibody. The whole antibody may be in any of the forms of the antibody isotypes eg IgG, IgA, IgE, and IgM and any of the forms of the isotype subclasses eg IgG1 or IgG4.

5       The specific binding member may also be in the form of an engineered antibody eg bispecific antibody molecules (or fragments such as  $F(ab')_2$ ) which have one antigen binding arm (ie specific binding domain) against  $TGF\beta$  and another arm against a different  
10       specificity. Indeed the specific binding members directed against  $TGF\beta 1$  and/or  $TGF\beta 2$  described herein may be combined in a bispecific diabody format. For example the antibodies 31G9 directed against  $TGF\beta 1$  and 6H1 directed against  $TGF\beta 2$  may be combined to give a  
15       single dimeric molecule with both specificities.

      The binding domain may comprise part or all of a VH domain encoded by a germ line gene segment or a rearranged gene segment. The binding domain may comprise part or all of either a VL kappa domain or a  
20       VL lambda domain.

      The binding domain may be encoded by an altered or variant form of a germ line gene with one or more nucleotide alterations (addition, deletion, substitution and/or insertion), e.g. about or less  
25       than about 25, 20, 15, 10 or 5 alterations, 4, 3, 2 or 1, which may be in one or more frameworks and/or CDR's.

      The binding domain may comprise a VH3 gene

sequence of one of the following germ lines; the DP49 germ line; the DP53 germ line; the DP50 germ line; the DP46 germ line; or a re-arranged form thereof.

A preferred VH domain for anti-TGF $\beta$ 2 specific  
5 binding members according to the present invention is that of 6H1 VH, whose sequence is shown in Figure 2(a) (i). 6H1 may be paired with a variety of VL domains, as exemplified herein. Amino acid sequence variants of 6H1 VH may be employed.

10 The specific binding member may neutralise the *in vitro* and/or *in vivo* effect of TGF $\beta$ , that is one or more of the isoforms, particularly TGF $\beta$ 1 and/or TGF $\beta$ 2.

The specific binding member may be a high affinity antibody. Preferred affinities are discussed  
15 elsewhere herein.

The binding domain may comprise part or all of a VH domain having either an amino acid sequence as shown in Fig 1(a) (i) or (ii) or Fig 1(c) (i) or a functionally equivalent variant form of a said amino  
20 acid sequence.

The binding domain may comprise part or all of a VH domain encoded by either a nucleotide sequence as shown in Fig 1(a) (i) or (ii) or Fig 1(c) (i) or a functionally equivalent variant form of a said  
25 nucleotide sequence.

The binding domain may comprise part or all of a VL domain having either an amino acid sequence as shown in Fig 1(a) (iii) or Fig 1(b) or a functionally

equivalent variant form of a said amino acid sequence.

The binding domain may comprise part or all of a VL domain encoded by either a nucleotide sequence as shown in Fig 1(a)(iii) or Fig 1(b) or a functionally  
5 equivalent variant form of a said nucleotide sequence.

The binding domain may comprise part or all of a VH domain having a variant form of the Fig 1(a)(i) amino acid, the variant form being one of those as provided by Fig 3.

10 The binding domain may comprise part or all of a VH domain having either an amino acid sequence as shown in Fig 2(a)(i) or (ii) or a functionally equivalent variant form of a said amino acid sequence.

The binding domain may comprise part or all of a  
15 VH domain encoded by either a nucleotide sequence as shown in Fig 2(a)(i) or (ii) or a functionally equivalent variant form of a said nucleotide sequence.

The binding domain may comprise part or all of a VL domain having either an amino acid sequence as  
20 shown in any of Figs 2(b)(i) to (v) or a functionally equivalent variant form of a said amino acid sequence.

The binding domain may comprise part or all of a VL domain encoded by either a nucleotide sequence as shown in any of Figs 2(b)(i) to (v) or a functionally  
25 equivalent variant form of a said nucleotide sequence.

The binding domain may be specific for both TGF $\beta$ 1 and TGF $\beta$ 2. The binding domain may be specific for both human TGF $\beta$ 1 and human TGF $\beta$ 2. The specific



binding member may be in the form of scFv.

The binding domain may comprise part or all of a VL domain having either an amino acid sequence as shown in Fig 4 or a functionally equivalent variant form of said amino acid sequence. The binding domain may comprise part or all of a VL domain encoded by either the nucleotide sequence as shown in Fig 4 or a functionally equivalent variant form of said nucleotide sequence.

10 In particular, the binding domain may comprise one or more CDR (complementarity determining region) with an amino acid sequence shown in any of the figures. In a preferred embodiment, the binding domain comprises one or more of the CDRs, CDR1, CDR2 and/or CDR3 shown in the Figures, especially any of those shown in Figure 19. In a preferred embodiment, the binding domain comprises a VH CDR3 sequence as shown, especially as shown in Figure 19. Functionally equivalent variant forms of the CDRs are encompassed by the present invention, in particular variants which differ from the CDR sequences shown by addition, deletion, substitution or insertion of one or more amino acids and which retain ability to bind the antigen and optionally one or more of the preferred characteristics for specific binding members of the present invention as disclosed herein. The specific binding member may comprise all or part of the framework regions shown flanking and between the CDRs

in the Figures, especially Figure 19, or different framework regions including modified versions of those shown.

So-called "CDR-grafting" in which one or more CDR sequences of a first antibody is placed within a framework of sequences not of that antibody, e.g. of another antibody is disclosed in EP-B-0239400.

The present invention also provides a polypeptide with a binding domain specific for TGF $\beta$  which polypeptide comprises a substantial part or all of either an amino acid sequence as shown in any of Fig 1(a), Fig 1(b), Fig 1(c), Fig 2(a), Fig 2(b), Fig 4 or a functionally equivalent variant form of a said amino acid sequence. The polypeptide may comprise a substantial part or all of an amino acid sequence which is a functionally equivalent variant form of the Fig 1(a)(i) amino acid sequence, the variant being one of those variants as shown in Fig 3.

Variable domain amino acid sequence variants of any of the VH and VL domains whose sequences are specifically disclosed herein may be employed in accordance with the present invention, as discussed. Particular variants may include one or more amino acid sequence alterations (addition, deletion, substitution and/or insertion), maybe less than about 20 alterations, less than about 15 alterations, less than about 10 alterations or less than about 5 alterations, 4, 3, 2 or 1. Alterations may be made in one or more

framework regions and/or one or more CDR's.

A specific binding member according to the invention may be one which competes for binding to TGF $\beta$ 1 and/or TGF $\beta$ 2 with any specific binding member  
5 which both binds TGF $\beta$ 1 and/or TGF $\beta$ 2 and comprises part of all of any of the sequences shown in the Figures. Competition between binding members may be assayed easily *in vitro*, for example by tagging a specific reporter molecule to one binding member which can be  
10 detected in the presence of other untagged binding member(s), to enable identification of specific binding members which bind the same epitope or an overlapping epitope.

Preferred specific binding members for TGF $\beta$ 1  
15 compete for binding to TGF $\beta$ 1 with the antibody CS37, discussed in more details elsewhere herein.

Preferred specific binding members for TGF $\beta$ 2 compete for binding to TGF $\beta$ 2 with the antibody 6B1 discussed in more detail elsewhere herein. They may  
20 bind the epitope RVLSL or a peptide comprising the amino acid sequence RVLSL, particularly such a peptide which adopts an  $\alpha$ -helical conformation. They may bind the peptide TQHSRVLSLYNTIN. In testing for this, a peptide with this sequence plus CGG at the N-terminus  
25 may be used. Specific binding members according to the present invention may be such that their binding for TGF $\beta$ 2 is inhibited by a peptide comprising RVLSL, such as a peptide with the sequence TQHSRVLSLYNTIN.

In testing for this, a peptide with this sequence plus CGG at the N-terminus may be used.

TQHSRVLSLYNTIN corresponds to the alpha helix H3 (residues 56-69) of TGF $\beta$ 2, as discussed elsewhere  
5 herein. The equivalent region in TGF $\beta$ 1 has the sequence TQYSKVLSLYNQHN. Anti-TGF $\beta$ 1 antibodies which bind this region are of particular interest in the present invention, and are obtainable for example by panning a peptide with this sequence (or with CGG at  
10 the N-terminus) against a phage display library. Specific binding members which bind the peptide may be selected by means of their binding, and may be neutralising for TGF $\beta$ 1 activity. Binding of such specific binding members to TGF $\beta$ 1 may be inhibited by  
15 the peptide TQYSKVLSLYNQHN (optionally with CGG at the N-terminus).

A specific binding member according to the present invention which is specific for TGF $\beta$ 2 may show no or substantially no binding for the latent form of  
20 TGF $\beta$ 2, i.e. be specific for the active form of TGF $\beta$ 2. 6B1 is shown in Example 6 to have this property.

6B1 is particularly suitable for therapeutic use in the treatment of fibrotic disorders because it has the following advantageous properties. 6B1 binds to  
25 TGF $\beta$ 2 with a dissociation constant of 2.3nM in the single chain form and 0.89nM for the whole antibody form, 6B1 IgG4 (Example 13). The antibody 6B1 IgG4 neutralises the biological activity of TGF $\beta$ 2 in an

antiproliferation assay ( $IC_{50}$  2nM; examples 7 and 10) and in a radioreceptor assay ( $IC_{50}$  less than 1nM; Table 6). The antibody binds to the peptide TQHSRVLSLYNTIN ( $TGF\beta 2_{56-69}$ ) from the alpha helix H3 of  $TGF\beta 2$  and  
5 recognises the corresponding peptide from  $TGF\beta 1$  more weakly. 6B1 recognises the active but not the latent form of  $TGF\beta 2$  (Example 6), recognises  $TGF\beta 2$  in mammalian tissues by ICC and does not bind non-specifically to other human tissues (Example 12). The  
10 antibody preferentially binds to  $TGF\beta 2$  as compared to  $TGF\beta 3$ , the cross-reactivity with  $TGF\beta 3$  being 9% as determined by the ratio of the dissociation constants.

The other antibodies described in this application which contain the 6H1 VH domain, 6H1 and  
15 6A5 have similar properties. The dissociation constants of were determined to be 2nM for 6B1 IgG4 (Example 2) and 0.7nM for 6A5 single chain Fv (Table 1). 6H1 IgG4 neutralises the biological activity of  $TGF\beta 2$  with  $IC_{50}$  values of 12 to 15nM (Examples 7 and  
20 10). 6A5 and 6H1 inhibit receptor binding of  $TGF\beta 2$  in a radioreceptor assay with  $IC_{50}$  values of about 1nM in the single chain Fv format and 10nM or below in the whole antibody, IgG4 format. Both 6H1 IgG4 and 6A5 scFv were shown to be effective in the prevention of  
25 neural scarring (Example 5).

Therefore for the first human antibodies directed against  $TGF\beta 2$  are provided which have suitable properties for treatment of diseases characterised by

the deleterious presence of TGF $\beta$ 2. Such antibodies preferably neutralise TGF $\beta$ 2 and preferably have a dissociation constant for TGF $\beta$ 2 of less than about 100nM, more preferably about 10nM, more preferably below about 5nM. The antibodies preferentially bind to TGF $\beta$ 2 as compared to TGF $\beta$ 3, preferably have less than 20% cross-reactivity with TGF $\beta$ 3 (as measured by the ratio of the dissociation constants) and preferably have less than about 10% cross-reactivity. The antibody preferably recognises the active but not the latent form of TGF $\beta$ 2.

For antibodies against TGF $\beta$ 1, the properties desired for an antibody to be effective in treatment of fibrotic disease are similar. Such antibodies preferably neutralise TGF $\beta$ 1 and have a dissociation constant for TGF $\beta$ 1 of less than about 100nM, more preferably below about 10nM, more preferably below about 5nM. The antibodies preferentially bind to TGF $\beta$ 1 as compared to TGF $\beta$ 3, preferably have less than about 20% cross-reactivity with TGF $\beta$ 3 (as measured by the ratio of the dissociation constants) and more preferably have less than about 10% cross-reactivity. The antibody preferably recognises the active but not the latent form of TGF $\beta$ 1. The antibody 31G9 has a dissociation constant of 12nM (Table 5). The antibodies CS37 scFv and 27C1/10A6 IgG4 show IC<sub>50</sub> values in a radioreceptor assay of 8nM and 9nM respectively, indicating a dissociation constant in

the low nanomolar range. 27C1/10A6 IgG4 was shown to be effective in a neural scarring model. Cross-reactivity of antibodies of the 1B2 lineage with TGF $\beta$ 3 is very low (Example 9).

5 In addition to an antibody sequence, the specific binding member may comprise other amino acids, e.g. forming a peptide or polypeptide, or to impart to the molecule another functional characteristic in addition to ability to bind antigen. For example, the specific  
10 binding member may comprise a label, an enzyme or a fragment thereof and so on.

The present invention also provides a polynucleotide which codes for a polypeptide with a binding domain specific for TGF $\beta$  which polynucleotide  
15 comprises a substantial part or all of a nucleotide sequence which codes for either an amino acid sequence as shown in any one of Fig 1(a), Fig 1(b), Fig 1(c), Fig 2(a), Fig 2(b), Fig 4 or a functionally equivalent variant form of a said amino acid sequence. The  
20 polynucleotide may code for a polypeptide with a binding domain specific for TGF $\beta$  which polynucleotide comprises a substantial part or all of a nucleotide sequence which codes for an amino acid sequence which is a functionally equivalent variant form of the Fig  
25 1(a)(i) amino acid sequence, the variant being one of those as shown in Fig 3. The polynucleotide may code for a polypeptide with a binding domain specific for TGF $\beta$  which polynucleotide comprises a substantial

part or all of a either a nucleotide sequence as shown  
in any of Fig 1(a), Fig 1(b), Fig 1(c), Fig 2(a), Fig  
2(b), Fig 4 or a functionally equivalent variant form  
of said nucleotide sequence. The polynucleotide may  
5 code for a polypeptide with a binding domain specific  
for TGF $\beta$  which polynucleotide comprises a substantial  
part or all a nucleotide sequence which codes for a  
variant form of the Fig 1(a)(i) amino acid sequence,  
the variant being one of those as shown in Fig 3.

10 The present invention also provides constructs in  
the form of plasmids, vectors, transcription or  
expression cassettes which comprise least one  
polynucleotide as above.

The present invention also provides a recombinant  
15 host cell which comprises one or more constructs as  
above.

A specific binding member according to the  
present invention may be made by expression from  
encoding nucleic acid. Nucleic acid encoding any  
20 specific binding member as provided itself forms an  
aspect of the present invention, as does a method of  
production of the specific binding member which method  
comprises expression from encoding nucleic acid  
therefor. Expression may conveniently be achieved by  
25 culturing under appropriate conditions recombinant  
host cells containing the nucleic acid. Following  
production by expression a specific binding member may  
be isolated and/or purified using any suitable



technique, then used as appropriate.

Specific binding members and encoding nucleic acid molecules and vectors according to the present invention may be provided isolated and/or purified, e.g. from their natural environment, in substantially pure or homogeneous form, or, in the case of nucleic acid, free or substantially free of nucleic acid or genes origin other than the sequence encoding a polypeptide with the required function. Nucleic acid according to the present invention may comprise DNA or RNA and may be wholly or partially synthetic. The term "isolate" encompasses all these possibilities.

The nucleic acid may encode any of the amino acid sequences shown in any of the Figures, or any functionally equivalent form. The nucleotide sequences employed may be any of those shown in any of the Figures, or may be a variant, allele or derivative thereof. Changes may be made at the nucleotide level by addition, substitution, deletion or insertion of one or more nucleotides, which changes may or may not be reflected at the amino acid level, dependent on the degeneracy of the genetic code.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, mammalian cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include

Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells and many others. A common, preferred bacterial host is *E. coli*.

The expression of antibodies and antibody fragments in prokaryotic cells such as *E. coli* is well established in the art. For a review, see for example Plückthun, A. *Bio/Technology* 9: 545-551 (1991).

Expression in eukaryotic cells in culture is also available to those skilled in the art as an option for production of a specific binding member, see for recent reviews, for example Reff, M.E. (1993) *Curr. Opinion Biotech.* 4: 573-576; Trill J.J. et al. (1995) *Curr. Opinion Biotech* 6: 553-560.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second

Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

Thus, a further aspect of the present invention  
5 provides a host cell containing nucleic acid as disclosed herein. A still further aspect provides a method comprising introducing such nucleic acid into a host cell. The introduction may employ any available technique. For eukaryotic cells, suitable techniques  
10 may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques  
15 may include calcium chloride transformation, electroporation and transfection using bacteriophage.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells under conditions for expression  
20 of the gene.

In one embodiment, the nucleic acid of the invention is integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote  
25 recombination with the genome, in accordance with standard techniques.

The present invention also provides a method which comprises using a construct as stated above in

an expression system in order to express a specific binding member or polypeptide as above.

Following production of a specific binding member it may be used for example in any of the manners disclosed herein, such as in the formulation of a composition, pharmaceutical or a diagnostic product, such as a kit comprising in addition to the specific binding member one or more reagents for determining binding of the member to cells, as discussed. A composition may comprise at least one component in addition to the specific binding member.

The present invention also provides pharmaceuticals which comprise a specific binding member as above, optionally with one or more excipients.

The present invention also provides the use of a specific binding member as above in the preparation of a medicament to treat a condition in which it is advantageous to counteract the fibrosis promoting effects of TGF $\beta$ . The condition may be a fibrotic condition characterized by an accumulation in a tissue of components of the extracellular matrix. The components of the extracellular matrix may be fibronectin or laminin.

The condition may be selected from the group consisting of: glomerulonephritis, neural scarring, dermal scarring, ocular scarring, lung fibrosis, arterial injury, proliferative retinopathy, retinal

detachment, adult respiratory distress syndrome, liver  
cirrhosis, post myocardial infarction, post  
angioplasty restenosis, keloid scarring, scleroderma,  
vascular disorders, cataract, glaucoma, proliferative  
5 retinopathy.

The condition may be neural scarring or  
glomerulonephritis.

The present invention also provides the use of a  
specific binding member as above, in the preparation  
10 of a medicament to treat an immune/inflammatory  
disease condition in which it is advantageous to  
counteract the effects of  $TGF\beta$ . Illustrative  
conditions are rheumatoid arthritis, macrophage  
deficiency disease and macrophage pathogen infection.

15 The present invention also provides a method  
which comprises administering to a patient a  
therapeutically effective amount of a specific binding  
member as above in order to treat a condition in which  
it is advantageous to counteract the fibrosis  
20 promoting effects of  $TGF\beta$ . Fibrotic conditions are  
listed above.

The present invention also provides a method  
which comprises administering to a patient a  
prophylactically effective amount of a specific  
25 binding member as above in order to prevent a  
condition in which it is advantageous to prevent the  
fibrosis promoting effects of  $TGF\beta$ . Fibrotic  
conditions are listed above.

The present invention also provides methods which comprise administering to patients prophylactically and/or therapeutically effective amounts of a specific binding member as above in order to prevent or treat  
5 an immune/inflammatory disease condition in which it is advantageous to counteract the effects of TGF $\beta$ . Illustrative conditions are stated above.

Thus, various aspects of the invention provide methods of treatment comprising administration of a  
10 specific binding member as provided, pharmaceutical compositions comprising such a specific binding member, and use of such a specific binding member in the manufacture of a medicament for administration, for example in a method of making a medicament or  
15 pharmaceutical composition comprising formulating the specific binding member with a pharmaceutically acceptable excipient.

In accordance with the present invention, compositions provided may be administered to  
20 individuals, which may be any mammal, particularly rodent, e.g. mouse, horse, pig, sheep, goat, cattle, dog, cat or human. Administration is preferably in a "therapeutically effective amount", this being sufficient to show benefit to a patient. Such benefit  
25 may be at least amelioration of at least one symptom. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription

of treatment, eg decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors. Appropriate doses of antibody are well known in the art; see Ledermann J.A. et al.

- 5 (1991) Int J. Cancer 47: 659-664; Bagshawe K.D. et al.  
(1991) Antibody, Immunoconjugates and  
Radiopharmaceuticals 4: 915-922.

A composition may be administered alone or in combination with other treatments, either  
10 simultaneously or sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may comprise, in addition to active  
15 ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature  
20 of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or  
25 liquid form. A tablet may comprise a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral

oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

5 For intravenous, injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are  
10 well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included,  
15 as required.

Further aspects of the invention and embodiments will be apparent to those skilled in the art. In order that the present invention is fully understood,  
20 the following examples are provided by way of exemplification only and not by way of limitation.

Reference is made to the following figures.

Figure 1 shows the DNA and protein sequences of  
25 antibodies specific for TGF $\beta$ 1. Figure 1(a) shows the amino acid and encoding nucleic acid sequences of antibody variable domains of antibodies to TGF $\beta$ 1 isolated directly from repertoires: Figure 1(a)(i)-



1B2 VH (also known as 7A3 VH); Figure 1(a) (ii) - 31G9 VH; Figure 1(a) (iii) - 31G9 VL. Figure 1 (b) shows the amino acid and encoding nucleic acid sequences of antibody light chain variable domains of antibodies to TGF $\beta$ 1 isolated by chain shuffling: Figure 1(b) (i) - 7A3 VL; Figure 1(b) (ii) - 10A6 VL. Figure 1(c) (i) shows the amino acid and encoding nucleic acid sequences for 27C1 VH, from an antibody to TGF $\beta$ 1 isolated from a CDR3 spiking experiment.

Figure 2 shows the DNA and protein sequences of antibodies specific for TGF $\beta$ 2. Figure 2(a) shows amino acid and encoding nucleic acid sequences for variable domains of antibodies to TGF $\beta$ 2 isolated directly from repertoires: Figure 2(a) (i) - 2A-H11 VH (also known as 6H1 VH); Figure 2(a) (ii) - 2A-A9 VH (also known as 11E6 VH). Figure 2(b) shows amino acid and encoding nucleic acid sequences of antibody variable domains of antibodies specific for TGF $\beta$ 2 isolated following chain shuffling: Figure 2(b) (i) - 6H1 VL; Figure 2(b) (ii) - 6A5 VL; Figure 2(b) (iii) - 6B1 VL; Figure 2(b) (iv) 11E6 VL; (v) Figure 2(b) (v) - 14F12 VL.

Figure 3 shows the protein sequences of VH CDR3 of clones derived from 1B2 by 'spiking' mutagenesis. Differences from 1B2 VH CDR3 are in bold.

Figure 4 shows the DNA and protein sequence of the VH and VL domains of VT37, cross-reactive between TGF $\beta$ 1 and TGF $\beta$ 2.

Figure 5 shows the DNA sequence and encoded amino acid sequence in the region of the heavy chain VH leader from the vector vhcassette2. Restriction enzymes HindIII, SfiI, PstI, BstEII, BamHI and EcoRI cut at the points indicated.

Figure 6 shows a map of the vector pG4D100 (not to scale). Multiple cloning site (MCS): 5'-HindIII-PacI-BamHI-(XanI)-(PmlI)-(NheI)-AscI-(BssHII)-XhoI-PmeI-BsiWI-3'. Restriction sites shown in brackets are not unique.

Figure 7 shows the DNA sequence, including intron, and encoded amino acid sequence in the region of the light chain VL leader for the vector vlcassettel (vlcassette CAT1). Restriction enzymes HindIII, ApaLI, SacI, XhoI and BamHI cut at the sites indicated (ApaLI within the leader).

Figure 8 shows a map of the vector pLN10 (not to scale). Multiple cloning site (MCS): 5'-HindIII-(SphI)-(PstI)-SalI-XbaI-BamHI-3' (1224-1259). Restriction sites shown in brackets are not unique.

Figure 9 shows a map of the vector pKN100 (not to scale). Multiple cloning site (MCS): 5'-MluI-(AvaI)-HindIII-(SphI)-(PstI)-SalI-XbaI-BamHI-3'. Restriction sites shown in brackets are not unique.

Figure 10 shows the % neutralisation of TGF $\beta$ 2 activity by single chain Fv antibodies in an assay using proliferation of the erythroleukaemia cell line TF1 at different nM concentrations of scFv.

Figure 11 shows the neutralisation of TGF $\beta$ 2 activity by whole IgG4 antibodies in an assay using proliferation of the erythroleukaemia cell line TF1 at different nM concentrations of antibody.

5        Figure 12 shows the effect of treatment of animals with antibodies on neural scarring as measured by the deposition of (Figure 12(a)) fibronectin and (Figure 12(b)) laminin detected using integrated fluorescence intensity. The graphs show scatter plots  
10 of individual animal data points. The bar graph shows the mean integrated fluorescence intensity of the group.

Figure 13 shows the results of an ELISA to measure the cross-reactivity of the antibodies 6B1  
15 IgG4 and 6A5 IgG4 with TGF $\beta$  isoforms and non-specific antigens. Figure 13(a) shows cross-reactivity of 6B1 IgG4 to a panel of non-specific antigens and TGF $\beta$ 's, plotting OD405nm for each antigen: 1 - interleukin 1; 2 - human lymphotoxin (TNF $\beta$ ); 3 - human insulin; 4 -  
20 human serum albumin; 5 - ssDNA; 6 - oxazolone-bovine serum albumin; 7 - keyhole limpet haemocyanin; 8 - chicken egg white trypsin inhibitor; 9 - chymotrypsinogen; 10 - cytochrome C; 11 - GADPH; 12 - ovalbumin; 13 - hen egg lysozyme; 14 - bovine serum  
25 albumin; 15 - TNF $\alpha$ ; 16 - TGF $\beta$ 1; 17 - TGF $\beta$ 2; 18 - TGF $\beta$ 3; 19 - PBS only. Figure 13(b) shows the OD405nm for the antibody 6A5 IgG4 against the same panel of antigens. For both Figure 13(a) and Figure 13(b),

antigens 1 to 15 were used for coating the plate at a concentration of 10 $\mu$ g/ml in PBS. The TGFbetas were coated at 0.2 $\mu$ g/ml in PBS. Coating was performed at 4°C overnight. 100 $\mu$ g of each antigen was used per well and duplicates of each antigen for each IgG to be tested. IgG samples were incubated with the coated antigens at 37°C for 2 hours after blocking with 2% marvel-PBS. The labelled second antibody was a mouse anti-human Fc1 alkaline phosphatase conjugated and the substrate used to detect bound second antibody was PNPP at 1mg/ml with the absorbance read at 405nm.

Figure 14 shows the amino acid and encoding nucleic acid sequence for the VL domain of the TGF $\beta$ 1-specific antibody CS37.

Figure 15 shows data from an ELISA detecting binding of 6B1 IgG4 to BSA conjugated with either peptide TGF $\beta$ 2<sub>56-69</sub> or peptide TGF $\beta$ 1<sub>56-69</sub> coated on to an ELISA plate. 6B1 IgG4 was incubated at various concentrations in  $\mu$ g/ml and the absorbance at 405nm measured after addition of the detection agents. OD405nm results are plotted at the various concentrations for BSA-TGF $\beta$ 2<sub>56-69</sub> ("Beta2 peptide" - diamonds) and BSA-TGF $\beta$ 1<sub>56-69</sub> ("Beta1 peptide" - squares).

Figure 16 shows % neutralisation of TGF- $\beta$ 2 anti-proliferative effect on TF1 cells by whole antibodies, 6H1 IgG4, 6B1 IgG4 and the mouse monoclonal from Genzyme, at various concentrations (nM IgG).

Figure 17 shows % neutralisation of TGF- $\beta$ 1 anti-proliferative effect on TF1 cells by whole antibodies, 6H1 IgG4, 6B1 IgG4 and the mouse monoclonal from Genzyme, at various concentrations (nM IgG).

5        Figure 18 shows % neutralisation of TGF- $\beta$ 3 anti-proliferative effect on TF1 cells by whole antibodies, 6H1 IgG4, 6B1 IgG4 and the mouse monoclonal from Genzyme, at various concentrations (nM IgG).

Figure 19 shows amino acid and encoding DNA  
10       sequences of regions of antibodies directed against TGF $\beta$ 2 showing CDR sequences in italics: Figure 19(i) 2A-H11 VH (also known as 6H1 VH); Figure 19(ii) 6B1 VL; Figure 19(iii) 6A5 VL and Figure 19(iv) 6H1 VL.

Figure 20 shows the vector p6H1 VH-gamma4 (7263  
15       bp). The gene encoding 6H1 VH is inserted as a HindIII-ApaI restriction fragment.

Figure 21 shows the vector p6B1 lambda (10151  
bp). The gene encoding 6B1 VL is inserted as an EcoRI-BstBI restriction fragment.

20       Figure 22 shows the vector p6B1 gamma4gs (14176 bp). The genes encoding the heavy and light chains of 6B1 IgG4 are combined in a single vector.

Figure 23 shows the results of competition ELISA experiments described in Example 6. Following  
25       overnight incubation with TGF $\beta$ 2, plates were treated with the following solutions 1-4 (number corresponding to those in Figure): 1 - 400 $\mu$ l Hams F12/DMEM (reagent blank), 2 - 400 $\mu$ l Hams F12/DMEM plus 4 $\mu$ g 6B1 IgG4

antibody (positive control), 3 - 400 $\mu$ l PC3 untreated conditioned media plus 4 $\mu$ g 6B1 IgG4 antibody (latent TGF $\beta_2$  sample), 4 - 400 $\mu$ l PC3 acid activated conditioned media plus 4 $\mu$ g 6B1 IgG4 antibody (active TGF $\beta_2$  sample).

All documents mentioned herein are incorporated by reference.

#### 10 List of Examples

Example 1 - Isolation of antibodies specific for TGF $\beta_1$ , antibodies specific for TGF $\beta_2$  and antibodies specific for TGF $\beta_1$  and TGF $\beta_2$ .

15 Example 2 - Construction of cell lines expressing whole antibodies.

Example 3 - Neutralisation of TGF $\beta$  activity by antibodies assessed using in vitro assays.

Example 4 - Inhibition by antibodies of TGF $\beta$  binding to receptors.

Example 5 - Prevention of neural scarring using antibodies against TGF $\beta$ .

Example 6 - Determination of Binding of 6B1 IgG4 to Active or Latent Form of TGF $\beta_2$ .

25 Example 7 - Neutralisation by antibodies directed against TGF $\beta_2$  of the inhibitory effect of TGF $\beta$  isoforms on cells proliferation.

Example 8 - Inhibition by antibodies directed

against TGF $\beta$ 2 of binding of other TGF $\beta$  isoforms to receptors measured in a radioreceptor assay.

Example 9 - Assessment of TGF $\beta$ 1 antibodies for potential therapeutic use.

5        Example 10 - Construction of a high expressing cell line for 6B1 IgG4 using the glutamine synthase selection system and assessment in a neutralisation assay.

10       Example 11 - Determination of the epitope on TGF $\beta$ 2 for the antibody 6B1 using a peptide phage display library.

Example 12 - Determination of the binding of 6B1 IgG4 to tissues by immunocytochemistry (ICC).

15       Example 13 - Determination of the kinetic parameters of 6B1 IgG4 and single chain Fv for binding to TGF $\beta$ 2.

Example 14 - Binding of a Peptide Corresponding to Residues 56 to 69 of TGF $\beta$ 2 to 6B1 IgG4.

## 20    *EXAMPLE 1*

*Isolation and Characterisation of Antibodies Binding to TGF $\beta$ 1 and TGF $\beta$ 2*

25    1 Identification and Characterisation of Antibodies to Human TGF $\beta$ -1 by Selection of Naive and Synthetic Phage Antibody Repertoires

*Antibody repertoires*

The following antibody repertoires were used:

1. Peripheral blood lymphocyte (PBL) library derived from unimmunized human (Marks, J. D., Hoogenboom, H. R. Bonnert, T. P., McCafferty, J., Griffiths, A. D. & Winter, G. (1991) J. Mol. Biol. 222, 581-597)
2. Synthetic library (Nissim, A., Hoogenboom, H. R., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D. and Winter, G. (1994) EMBO J. 13, 692-698) derived from cloned human germline VH genes and synthetic CDR3s with a fixed light chain
3. Tonsil library derived from the tonsils of unimmunised humans. Tonsil B cells were isolated from freshly removed (processed within 2 hours) whole tonsils provided by Addenbrookes Hospital, Hills Road, Cambridge, U.K. Each tonsil was processed as follows. Tonsils were placed in a petri dish containing 5ml of PBS and macerated with a scalpel blade to release the cells. The suspension was transferred to a fresh tube and large debris allowed to sediment under gravity for 5 minutes. The cell suspension was then overlaid onto 10mls of Lymphoprep in a 50 ml polypropylene tube (Falcon) and centrifuged at 1000xg 20 minutes at room temperature (no brake) and cells at the interface harvested with a glass pipette. These were diluted to a final volume of 50 ml in RPMI medium at 37<sup>0</sup> C and



centrifuged at 500xg for 15 minutes at room temperature. The supernatant was aspirated and the cells washed another two times with RPMI.

Polyadenylated RNA was prepared from pelleted cells using the "Quickprep<sup>TM</sup> mRNA Kit" (Pharmacia Biotech, Milton Keynes, U.K.). The entire output of cells from one tonsil (ca.  $1 \times 10^6$  cells) was processed using one Oligo(dT)-Cellulose Spun column and processed exactly as described in the accompanying protocol. MRNA was ethanol precipitated as described and resuspended in 40ml RNase free water.

The cDNA synthesis reaction was set up using the "First-Strand cDNA Synthesis Kit" (Pharmacia Biotech, Milton Keynes, U.K.) as follows:

|    |                           |   |
|----|---------------------------|---|
| 15 | RNA                       | 20 $\mu$ l (heated to 67°C 10 minutes before use) |
|    | 1st strand buffer         | 11 $\mu$ l  |
|    | DTT solution              | 1 $\mu$ l   |
|    | pd(N) <sub>6</sub> primer | 1 $\mu$ l   |

20 After gentle mixing, the reaction was incubated at 37°C for 1 hour.

Human VH genes were amplified from tonsil cDNA using the nine family-based back primers (VH 1b/7a -6a back Sfi , which introduce a Sfi I site at the 5'-end, Table 1) together with an equimolar mixture of the four JH forward primers (JH 1-2, 3, 4-5, 6, for; Marks et al., 1991 supra). Thus, nine primary PCR amplifications were performed. Each reaction mixture

(50  $\mu$ l) comprised 2  $\mu$ l cDNA template, 25 pmol back primer, 25 pmol forward primers, 250  $\mu$ M dNTPs, 1.5 mM  $MgCl_2$ , 50 mM KCl, 10 mM Tris-HCL pH 8.3 and 2.5 u of Taq polymerase (Boehringer). The reaction mixture was overlaid with mineral (paraffin) oil and was cycled 30 times (94  $^{\circ}$ C for 1 min, 55  $^{\circ}$ C for 1 min, 72  $^{\circ}$ C for 1 min) using a Techne thermal cycler. The products were purified on a 1% (w/v) agarose gel, isolated from the gel using "Geneclean" (Bio 101 Inc.) and resuspended in 15  $\mu$ l of water. The amplified VH genes were recombined with human VL genes derived from PBLs (Marks et al., 1991 supra) together with the (Gly<sub>4</sub>, Ser)<sub>3</sub> linker (Huston, J.S., et al. 1988 Proc Natl Acad Sci U S A. 85: 5879-83) by PCR assembly (Marks et al., 1991 supra). The VH-linker-VL antibody constructs were cloned into the SfiI and NotI sites of the phagemid vector, pCANTAB6 (McCafferty, J., et al. 1994 Appl. Biochem. Biotech. 47: 157 - 173) to give a library of  $6 \times 10^7$  clones.

20

4. Large single chain Fv library derived from lymphoid tissues including tonsil, bone marrow and peripheral blood lymphocytes.

Polyadenylated RNA was prepared from the B-cells of various lymphoid tissues of 43 non-immunised donors using the "Quickprep mRNA Kit" (Pharmacia). First-strand cDNA was synthesized from mRNA using a "First-strand cDNA synthesis" kit (Pharmacia) using

random hexamers to prime synthesis. V-genes were amplified using family-specific primers for VH, V $\kappa$  and V $\lambda$  genes as previously described (Marks et al., supra) and subsequently recombined together with the (Gly<sub>4</sub>, Ser)<sub>3</sub> scFv linker by PCR assembly. The VH-linker-VL antibody constructs were cloned into the Sfi I and Not I sites of the phagemid vector, pCANTAB 6. Ligation, electroporation and plating out of the cells was as described previously (Marks et al., 1991 supra). The library was made ca. 1000x larger than that described previously by bulking up the amounts of vector and insert used and by performing multiple electroporations. This generated a scFv repertoire that was calculated to have ca.  $1.3 \times 10^{10}$  individual recombinants which by Bst NI fingerprinting were shown to be extremely diverse.

*a. Induction of phage antibody libraries*

The four different phage antibody repertoires above were selected for antibodies to TGF $\beta$ -1. The VH synthetic (Nissim et al., 1994 supra), tonsil, 'large' scFv and PBL (Marks et al., 1991 supra) repertoires were each treated as follows in order to rescue phagemid particles. 500 ml prewarmed (37 °C) 2YTAG (2YT media supplemented with 100  $\mu$ g/ml ampicillin and 2 % glucose) in a 2 l conical flask was inoculated with approximately  $3 \times 10^{10}$  cells from a glycerol stock (-70 °C) culture of the appropriate library. The

culture was grown at 37 °C with good aeration until the OD<sub>600nm</sub> reached 0.7 (approximately 2 hours). M13K07 helper phage (Stratagene) was added to the culture to a multiplicity of infection (moi) of approximately 10 (assuming that an OD<sub>600nm</sub> of 1 is equivalent to 5 x 10<sup>8</sup> cells per ml of culture). The culture was incubated stationary at 37 °C for 15 minutes followed by 45 minutes with light aeration (200 rpm) at the same temperature. The culture was centrifuged and the supernatant drained from the cell pellet. The cells were resuspended in 500 ml 2YTAK (2YT media supplemented with 100 µg/ml ampicillin and 50 µg/ml kanamycin), and the culture incubated overnight at 30 °C with good aeration (300 rpm). Phage particles were purified and concentrated by three polyethylene glycol (PEG) precipitations (Sambrook, J., Fritsch, E.F., & Maniatis, T. (1990). Molecular Cloning - A Laboratory Manual. Cold Spring Harbour, New York) and resuspended in PBS to 10<sup>12</sup> transducing units (tu)/ml (ampicillin resistant clones).

*b. Panning of phage antibody library on TGFβ-1*

Phage induced from the four repertoires were each separately panned on TGFβ-1. A 75mm x 12mm immuno tube (Nunc; Maxisorp) was coated with 2 ml of recombinant human TGFβ-1 (0.5ug/ml, Genzyme) in PBS overnight at 4 °C. After washing 3 times with PBS, the tube was

filled with 3%MPBS (3 % 'Marvel' skimmed milk powder, 1x PBS) and incubated for 2 hours at 37 °C for blocking. The wash was repeated, phagemid particles ( $10^{13}$  tu) in 2 ml of 3% MPBS were added and the tube  
5 incubated stationary at 37 °C for 1 hour. The tube was washed 20 times with PBST(0.1%), then 20 times with PBS. Bound phage particles were eluted from the tube by adding 2 ml of 100mM-triethylamine, and incubating the tube stationary at room temperature for  
10 10 minutes. The eluted material was immediately neutralised by pipetting into a tube containing 1 ml 1M-Tris.HCl (pH7.4). Phage were stored at 4 °C. 1.5 ml of the eluted phage were used to infect 20 ml of logarithmically growing E. coli TG1 (Gibson, T.J.  
15 (1984). PhD thesis. University of Cambridge, UK.). Infected cells were grown for 1 hour at 37 °C with light aeration in 2YT broth, and then plated on 2YTAG medium in 243mm x 243mm dishes (Nunc). Plates were incubated overnight at 30 °C. Colonies were scraped  
20 off the plates into 10 ml of 2YT broth and 15 % (v/v) glycerol added for storage at -70 °C.

Glycerol stock cultures from the first round of panning of each of the four repertoires on TGF $\beta$ -1 were each rescued using helper phage to derive phagemid  
25 particles for the second round of panning. 250  $\mu$ l of glycerol stock was used to inoculate 50 ml 2YTAG broth, and incubated in a 250 mL conical flask at 37 °C with good aeration until the OD<sub>600nm</sub> reached 0.7

(approximately 2 hours). M13K07 helper phage (moi=10) was added to the culture which was then incubated stationary at 37 °C for 15 minutes followed by 45 minutes with light aeration (200 rpm) at the same temperature. The culture was centrifuged and the supernatant drained from the cell pellet. The cells were resuspended in 50 ml prewarmed 2YTAK, and the culture incubated overnight at 30 °C with good aeration. Phage particles were purified and concentrated by PEG precipitation (Sambrook et al., 1990 supra) and resuspended in PBS to 1013 tu/ml.

Phage induced from the first round of panning of each of the three repertoires, was selected a second time essentially as described above except that the panning tube was coated with only 1 ml of TGF $\beta$ -1 (0.5ug/ml, Genzyme), and the volume of phage added to the tube similarly reduced. After extensive washing, bound phage were eluted from the tube using 1 ml of 100 mM-triethylamine, and neutralised by the addition of 0.5 ml 1M-Tris.HCl (pH7.4) as earlier described. The process of phage growth and panning was repeated over a third and a fourth round of selection.

*c. Growth of single selected clones for immunoassay*

Individual colonies from the third and fourth round selections were used to inoculate 100  $\mu$ l 2YTAG into individual wells of 96 well tissue culture plates (Corning). Plates were incubated at 30 °C overnight

with moderate shaking (200 rpm). Glycerol to 15 % was added to each well and these master plates stored at  $-70^{\circ}\text{C}$  until ready for analysis.

5    *d. ELISA to identify anti-TGF $\beta$ -1 scFv*

Clones specific for TGF $\beta$ -1 were identified by ELISA, using scFv displayed on phage or soluble scFv.

i. Phage ELISA

10        Cells from the master plates were used to inoculate fresh 96 well tissue culture plates containing 100  $\mu\text{l}$  2YTAG per well. These plates were incubated at  $37^{\circ}\text{C}$  for 6-8 hours or until the cells in the wells were growing logarithmically (OD600  
15    0.2-1.0). M13K07 was added to each well to an moi of 10 and incubated stationary for 15 min then 45 min with gentle shaking (100 rpm), both at  $37^{\circ}\text{C}$ . The plates were centrifuged at 2000 rpm for 10 min and the supernatant eluted. Each cell pellet was resuspended  
20    in 100  $\mu\text{l}$  2YTAK and incubated at  $30^{\circ}\text{C}$  overnight.

Each plate was centrifuged at 2000 rpm and the 100  $\mu\text{l}$  supernatant from each well recovered and blocked in 20  $\mu\text{l}$  18%M6PBS (18 % skimmed milk powder, 6 x PBS), stationary at room temperature for 1 hour.

25    Meanwhile, flexible microtitre plates which had been blocked overnight stationary at  $4^{\circ}\text{C}$  with either 50  $\mu\text{l}$  0.2  $\mu\text{g/ml}$  TGF $\beta$ -1 in PBS or 50  $\mu\text{l}$  PBS alone (giving an uncoated control plate), were washed 3 times in PBS

and blocked for 2 h stationary at 37 °C in 3MPBS. These plates were then washed three times with PBS and 50 µl preblocked phage added to each well of both the TGFβ-1-coated or uncoated plate. The plates were  
5 incubated stationary at 37 °C for 1 h after which the phage were poured off. The plates were washed by incubating for 2 min in PBST three times followed by incubating for 2min in PBS three times, all at room temperature.

10 To each well of both the TGFβ-1-coated and the uncoated plate, 50 µl of a 1 in 10,000 dilution of sheep anti-fd antibody (Pharmacia) in 3MPBS was added and the plates incubated at 37 °C stationary for 1 h. Each plate was washed as described above and 50 µl of  
15 a 1 in 5,000 dilution donkey anti-sheep alkaline phosphatase conjugate (Sigma) in 3MPBS added and incubated stationary at 37 °C for 1 h. Plates were washed as described as above followed by two rinses in 0.9% NaCl. Alkaline phosphatase activity was  
20 visualised using either the chromagenic substrate pNPP (Sigma) or the Ampak system (Dako). The absorbance signal generated by each clone was assessed by measuring the optical density at either 405 nm (pNPP) or 492 nm (Ampak) using a microtitre plate reader.  
25 Clones were chosen for further analysis if the ELISA signal generated on the TGFβ-1-coated plate was at least double that on the uncoated plate.



ii. Soluble ELISA

Cells from the master plates were used to inoculate fresh 96 well tissue culture plates containing 100  $\mu$ l 2YTAG per well. These plates were  
5 incubated at 30 °C for 8 hours then centrifuged at 2000 rpm for 10 min and the supernatant eluted. Each cell pellet was resuspended in 100  $\mu$ l 2YTA ( 2YT media supplemented with 100ug/ml ampicillin) containing 10 mM IPTG ( isopropyl-B-D-thiogalactopyranoside) and  
10 incubated at 30 °C overnight.

Each plate was centrifuged at 2000 rpm and the 100  $\mu$ l supernatant from each well recovered and blocked in 20  $\mu$ l 18%M6PBS stationary at room temperature for 1 hour. Meanwhile, flexible  
15 microtitre plates which had been blocked overnight stationary at 4 °C with either 50  $\mu$ l 0.2  $\mu$ g/ml TGF $\beta$ -1 in PBS or 50  $\mu$ l PBS alone, were washed 3 times in PBS and blocked for 2 h stationary at 37 °C in 3%MPBS. These plates were then washed three times with PBS and  
20 50  $\mu$ l preblocked soluble scFv added to each well of both the TGF $\beta$ -1-coated or uncoated plate. The plates were incubated stationary at 37 °C for 1 h after which the scFv solutions were poured off. The plates were washed by incubating for 2 min in PBST ( PBS  
25 containing 1% Tween) three times followed by incubating for 2 min in PBS three times, all at room temperature.

To each well of both the TGF $\beta$ -1-coated and the

uncoated plate, 50  $\mu$ l of a 1 in 200 dilution of the anti-myc tag murine antibody 9E10 (Munro, S. & Pelham, H.R.B. (1986) Cell 46, 291-300) in 3MPBS was added and the plates incubated at 37  $^{\circ}$ C stationary for 1 h.

- 5 Each plate was washed as described above and 50  $\mu$ l of a 1 in 5,000 dilution goat anti-mouse alkaline phosphatase conjugate (Pierce) in 3MPBS added and incubated stationary at 37  $^{\circ}$ C for 1 h. Plates were washed as described above followed by two rinses in
- 10 0.9% NaCl. Alkaline phosphatase activity was visualised using either the chromagenic substrate pNPP (Sigma) or the Ampak system (Dako). The absorbance signal generated by each clone was assessed by measuring the optical density at either 405 nm (pNPP)
- 15 or 492 nm (Ampak) using a microtitre plate reader. Clones were chosen for further analysis if the ELISA signal generated on the TGF $\beta$ -1-coated plate was at least double that on the uncoated plate.

20 iii. Specificity ELISA

- Clones identified as binding TGF $\beta$ -1 rather than uncoated well, as described above, were further analysed for fine specificity. Specificity ELISA's were carried out using scFv either displayed on phage
- 25 or in solution as described above, except that 5 ml of media in 50 ml Falcon tubes were inoculated with each clone and grown to generate the phage or soluble scFv used in the ELISA. Microtitre plate wells were coated

with 50  $\mu$ l of either 0.2  $\mu$ g/ml TGF $\beta$ -1, 0.2  $\mu$ g/ml TGF $\beta$ -2, 10  $\mu$ g/ml bovine serum albumin (BSA) or PBS (the uncoated well). After preblocking both the phage (or soluble scFv) and the microtitre plates, 50  $\mu$ l  
5 blocked phage (or soluble scFv) from each clone was added to a well coated with either TGF $\beta$ -1, TGF $\beta$ -2, BSA or an uncoated well. As above, alkaline phosphatase activity was visualised using either the chromagenic substrate pNPP (Sigma) or the Ampak system  
10 (Dako). Clones were considered to be specific for TGF $\beta$ -1 if the ELISA signal generated in the TGF $\beta$ -1 coated well was at least five-fold greater than the signal on either TGF $\beta$ -2, BSA or an uncoated well.

15 iv. Specificity determination by BIAcore™

The antibodies were also shown to be specific for TGF $\beta$ 1 compared to TGF $\beta$ 2 (obtained from R&D Systems Abingdon) by relative binding to the BIAcore™ sensor chips coated with the appropriate antigen. TGF $\beta$ 1 and  
20 TGF $\beta$ 2 were immobilised by amine coupling to Biosensor CM5 sensorchips (Pharmacia) according to the manufacturers instructions. Single chain Fv fragments (35 $\mu$ l; purified by immobilized metal affinity chromatography as described in example 4) were  
25 injected over the immobilized antigen at a flow rate of 5 $\mu$ l/min. The amount of TGF $\beta$  bound was assessed as the total increase in resonance units (RUs) over this period. For 31G9 scFv an increase of 1059RUs was found

with a TGF $\beta$ 1 chip and 72 RUs was found with a TGF $\beta$ 2 chip. Thus binding is much stronger to TGF $\beta$ 1 than TGF $\beta$ 2.

5    *e. Sequencing of TGF $\beta$ 1-Specific ScFv Antibodies*

The nucleotide sequence of the TGF $\beta$ -1 specific antibodies was determined by first using vector-specific primers to amplify the inserted DNA from each clone. Cells from an individual colony on  
10 a 2YTAG agar plate were used as the template for a polymerase chain reaction (PCR) amplification of the inserted DNA using the primers pUC19reverse and fdtetseq (Table 1). Amplification conditions consisted of 30 cycles of 94 °C for 1 min, 55 °C for 1  
15 min and 72 °C for 2 min, followed by 10 min at 72 °C. The PCR products were purified using a PCR Clean-up Kit (Promega) in to a final volume of 50  $\mu$ l H<sub>2</sub>O. Between 2 and 5  $\mu$ l of each insert preparation was used as the template for sequencing using the Taq  
20 Dye-terminator cycle sequencing system (Applied Biosystems). The primers mycseq10 and PCR-L-Link were used to sequence the light chain of each clone and PCR-H-Link and pUC19reverse to sequence the heavy chain (Table 1)

25

*f. Sequence and Source of the Initial TGF $\beta$ -1-Specific ScFv Antibodies*

Four different TGF $\beta$ -1 specific antibodies were

isolated from the selections using the four libraries described above. Each clone name, its origin and its heavy and light chain germline is given below. The complete sequence of the VH domain genes of clones 1-B2 and 31-G9 are given in Figure 1(a) together with the VL domain gene from scFv 31-G9.

|    | <u>CLONE</u> | <u>LIBRARY SOURCE</u> | <u>VH GERMLINE</u> | <u>VL ISOTYPE</u> |
|----|--------------|-----------------------|--------------------|-------------------|
| 10 | 1-B2         | PBL                   | VH3 DP49           | VKappa            |
|    | 1A-E5        | Synthetic VH          | VH3 DP53           | VLambda           |
|    | 1A-H6        | Tonsil                | VH3 DP50           | VLambda           |
|    | 31-G9        | large scFv            | VH3 DP49           | VLambda           |

Thus these initial isolates were obtained from libraries derived from different sources-both natural V genes of unimmunised humans and synthetic libraries from cloned germline V genes together with synthetic CDRs.

20

## 2. Affinity Maturation of the Initial TGF $\beta$ -1-Specific ScFv Antibodies

### *a. Light Chain Shuffling of the TGF $\beta$ -1-Specific ScFv*

25 Antibody 1-B2

#### i. Construction of Repertoires

The heavy chain of clone 1-B2 was recombined with

the complete repertoire of light chains derived from the PBL and large (tonsil-derived) scFv repertoires. The 1-B2 heavy chain was amplified by PCR using the primers HuJh4-5For (Table 1) and pUC19reverse.

5 Amplification conditions consisted of 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1min, followed by 10 min at 72 °C. The PCR product was separated through a 1% agarose-TAE gel, the band representing the amplified VH excised, and eluted from  
10 the agarose gel using the GeneClean Kit (Bio 101).

The PBL and tonsil light chains were amplified by PCR using the primers fdtetseq and a mix of RL1, 2 & 3 (Table 1). Amplification conditions consisted of 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C  
15 for 1min, followed by 10 min at 72 °C. The PCR product was separated through a 1% agarose-TAE gel, the band representing the amplified VL excised, and eluted from the agarose gel using the GeneClean Kit (Bio 101).

20 Approximately 50 ng amplified 1-B2 heavy chain and 50 ng of either amplified PBL-derived or amplified tonsil-derived light chains were combined and precipitated with sodium acetate and ethanol using 25 µg glycogen as a carrier. The precipitated DNA was  
25 pelleted by centrifugation at 13,000 rpm in a microfuge, air dried and resuspended in 26 µl H2O. This was used in an assembly amplification after the addition of reaction buffer to 1X, dNTP's to 200 nM

and 5 units Taq polymerase. Amplification conditions consisted of 20 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1min 30 s, followed by 10 min at 72 °C. 10 µl of each assembly was used as the template in a 'pull-through' amplification with the primers fdtetseq and pUC19reverse. Amplification conditions consisted of 25 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1min 30 s, followed by 10 min at 72 °C.

10       The pull-through amplification product was separated through 1% agarose-TAE and the band representing the pull-through VH-VL excised and eluted using the GeneClean Kit. This was digested with the restriction endonucleases Sfi I and Not I (NEB) and  
15       ligated (Amersham ligation system) into the phagemid vector pCantab 6, previously digested with Sfi I and Not I. The ligation product was used to transform electrocompetent TG1 cells, plated out on 2YTAG plates and incubated overnight at 30 °C. Approximately 1 x  
20       10<sup>5</sup> individual clones were generated from the light chain-shuffle of the 1-B2 heavy chain with the PBL-derived light chains and approximately 1 x 10<sup>6</sup> for the shuffle with the tonsil-derived light chains.

25       ii. Selection of Light Chain Shuffle Repertoires

      The two light chain-shuffle repertoires were selected for TGFβ-1-specific antibodies. Phagemid particles were recovered from each repertoire as

described earlier for the initial libraries.

Recovered phage were preblocked for 1 h in a final volume of 100  $\mu$ l 3MPBS. Approximately  $10^{11}$  tu phage were used in the first round selection and between  $10^9$  and  $10^{10}$  for subsequent selections. For the first round selections, biotinylated TGF $\beta$ 1 to a final concentration of 100 nM was added to the preblocked phage and incubated stationary at 37°C for 1h.

For each selection, 100  $\mu$ l Dynabeads suspension (Dyna) was separated on a magnet and the beads recovered and preblocked for 2 h in 1 ml 3MPBS. The beads were recovered on a magnet and resuspended in the phagemid/biotinylated TGF $\beta$ -1 mixture and incubated at room temperature for 15 min while being turned end-over-end. The beads were captured on a magnet and washed four times with PBST followed by three washes in PBS. After each wash, the beads were captured on a magnet and resuspended in the next wash. Finally, half of the beads were resuspended in 10  $\mu$ l 50 mM DTT (the other half of the beads stored at 4 °C as a back-up) and incubated at room temperature for 5 min. The whole bead suspension was then used to infect 5 ml logarithmically-growing TG1 cells. This was incubated at 37 °C, stationary for 15 min then with moderate shaking for 45 min, plated on 2YTAG plates and incubated overnight at 30 °C.

Colonies were scraped off the plates into 10 ml of 2YT broth and 15 % (v/v) glycerol added for storage



at -70 °C. A 250 µl aliquot of each plate scrape was used to inoculate 2YTAG and phagemid particles rescued as described earlier. For each repertoire, three rounds of selection using biotinylated TGFβ-1 was performed, essentially identical to the first round selection described above. All selections were at 100 nM TGFβ-1 except for the third round selection of the tonsil-derived light chain repertoire where the concentration of biotinylated TGFβ-1 in the selection was reduced to 50 nM.

iii. Identification of TGFβ-1-Specific ScFv Antibodies from Light Chain Shuffle Repertoires

ScFv antibodies specific to TGFβ-1 were identified by both phage and soluble ELISA, and sequenced, as described earlier. Three new TGFβ-1-specific scFv antibodies were identified, two with PBL-derived light chains and one with a tonsil-derived light chain. All three had the 1B2 heavy chain sequence (DP49), described earlier. The sequences are summarised below and the complete sequence of each VL domain gene is given in figure 1(b).

| CLONE | VL SOURCE | VH GERMLINE | VL ISOTYPE |
|-------|-----------|-------------|------------|
| 7-A3  | PBL       | DP49 (1B2)  | VKappa     |
| 10-A6 | PBL       | DP49 (1B2)  | VLambda    |

14-A1      Tonsil      DP49 (1B2)      VLambda

Thus the VH domain 1B2 derived from the PBL library can be combined with VL domains derived from  
5 both PBL and tonsil libraries.

*b. CDR3 'Spiking' of the TGF $\beta$ -1-Specific ScFv Antibody 1B2*

10 i. Construction of 'spiked' repertoire

An 84 mer mutagenic oligonucleotide primer, 1B2 mutVHCDR3, was first synthesized (see Table 1). This primer was 'spiked' at 10%; i.e. at each nucleotide position there is a 10% probability that a  
15 non-parental nucleotide will be incorporated. The 1-B2 heavy chain was amplified by PCR using the primers pUC19reverse and 1B2 mutVHCDR3. Amplification conditions consisted of 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1min, followed by 10 min  
20 at 72 °C. The PCR product was separated through a 1% agarose-TAE gel, the band representing the amplified VH excised, and eluted from the agarose gel using the Geneclean Kit (Bio 101).

The parental 1B2 light chain was amplified by PCR  
25 using the primers fdtetseq and RL3 (Table 1). Amplification conditions consisted of 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1min, followed by 10 min at 72 °C. The PCR product was

separated through a 1% agarose-TAE gel, the band representing the amplified VL excised, and eluted from the agarose gel using the Geneclean Kit (Bio 101).

Approximately 50 ng amplified 'spiked' 1-B2 heavy  
5 chain and 50 ng of amplified parental 1B2 light chain  
were combined and precipitated with sodium acetate and  
ethanol using 25 µg glycogen as a carrier. The  
precipitated DNA was pelleted by centrifugation at  
13,000 rpm in a microfuge, air dried and resuspended  
10 in 26 µl H<sub>2</sub>O. This was used in an assembly  
amplification after the addition of reaction buffer to  
1X, dNTP's to 200 nM and 5 units Taq polymerase.  
Amplification conditions consisted of 25 cycles of 94  
°C for 1 min, 65 °C for 4 min. Five µl of each  
15 assembly was used as the template in a 'pull-through'  
amplification with the primers fdtetseq and  
pUC19reverse. Amplification conditions consisted of  
30 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C  
for 1min, followed by 10 min at 72 °C.

20 The pull-through amplification product was  
separated through 1% agarose-TAE and the band  
representing the pull-through 'spiked' VH -VL excised  
and eluted using the Geneclean Kit. This was digested  
with the restriction endonucleases Sfi I and Not I  
25 (NEB) and ligated (Amersham ligation system) into the  
phagemid vector pCantab 6, previously digested with  
Sfi I and Not I. The ligation product was used to  
transform electrocompetent TG1 cells, plated out on

2YTAG plates and incubated overnight at 30 °C.

Approximately  $4 \times 10^6$  individual clones were generated from this VH CDR3 'spiking' of the 1-B2 VH CDR3.

5    ii. Selection of 1B2 CDR3 Spike Repertoire

          The repertoire was selected for new  
TGFβ-1-specific scFv antibody by one round of panning  
on 1 µg/ml TGFβ-1 followed by two rounds of selection  
with biotinylated TGFβ-1 at 50 nM using methods as  
10    described earlier.

iii. Identification of TGFβ-1-Specific ScFv Antibodies  
from the 1B2 CDR3 Spike Repertoire

          ScFv antibodies specific to TGFβ-1 were  
15    identified by both phage and soluble and phage ELISA,  
and sequenced, as described earlier. Clone 27C1 was  
isolated from the spiked repertoire. It is virtually  
identical to clone 1B2 but with three differences in  
the heavy chain CDR3. The complete sequence of clone  
20    27C1 is given in figure 1 (c). The 27C1 VH domain was  
combined with the 10A6 VL domain in the construction  
of the whole antibody 27C1/10A6 IgG4 (example 2). The  
properties of this antibody are described in more  
detail in examples 2 to 6. In addition to 27C1, a  
25    large number of other antibodies were isolated with up  
to 7 of the 14 amino acids differing in CDR3 of the VH  
domain (Figure 3). These had a similar preference for  
binding TGFβ1 compared to TGFβ2.

3. *Identification and Characterisation of Antibodies to Human TGF $\beta$ -2 by Selection of Naive and Synthetic Phage Antibody Repertoires*

5 a. Induction of phage antibody libraries

Two different phage antibody repertoires were selected for antibodies to TGF $\beta$ -2. The VH synthetic (Nissim et al., 1994) and tonsil (constructed as described earlier) repertoires were each treated as  
10 described for TGF $\beta$ -1 to rescue phagemid particles.

b. Panning of phage antibody library on TGF $\beta$ -2

Phage induced from the two repertoires were each separately panned on TGF $\beta$ -2 as described earlier for  
15 TGF $\beta$ -1 but using 0.5  $\mu$ g/ml TGF $\beta$ -2 as the coating antigen.

c. Identification and Sequencing of TGF $\beta$ -2-Specific ScFv Antibodies

20 Individual colonies from the third and fourth round selections were screened by both phage and soluble ELISA as described earlier for TGF $\beta$ -1 but using flexible microtitre plates coated with TGF $\beta$ -2 at 0.2  $\mu$ g/ml rather than TGF $\beta$ -1. Clones were chosen for  
25 further analysis if the ELISA signal generated on the TGF $\beta$ -2-coated plate was at least double that on the uncoated plate. For the specificity ELISA, as described earlier for TGF $\beta$ -1, clones were considered

to be specific for TGF $\beta$ -2 if the ELISA signal generated in the TGF $\beta$ -2 coated well was at least five-fold greater than the signal on either TGF $\beta$ -1, BSA or an uncoated well.

5

d. Sequence and Source of the Initial TGF $\beta$ -2-Specific ScFv Antibodies

Four different TGF $\beta$ -2 specific antibodies were isolated from the selections using the two libraries described above. Each clone name, its origin and its heavy and light chain germline is given below. The complete sequence of the VH domain genes of 2A-H11 and 2A-A9 are given in Figure 2 (a).

|    |              |                       |                    |                   |
|----|--------------|-----------------------|--------------------|-------------------|
| 15 | <u>CLONE</u> | <u>LIBRARY SOURCE</u> | <u>VH GERMLINE</u> | <u>VL ISOTYPE</u> |
|    | 1-G2         | Tonsil                |                    |                   |
|    | 1-H6         | Tonsil                | DP49               |                   |
|    | 2A-H11       | Synthetic VH          | DP50               | VLambda           |
| 20 | 2A-A9        | Synthetic             | DP46               | VLambda           |
|    | Gold-11      | Large scFv            |                    | VLambda           |

Thus human antibodies binding to human TGF $\beta$ 2 have been isolated from different sources-, both natural Vgenes of unimmunised humans and synthetic libraries from cloned germline V genes together with synthetic CDRs.

25

4. *Light Chain Shuffling of the TGF $\beta$ -2-Specific ScFv Antibodies 2A-H11 and 2A-A9*

a. Construction of Repertoires

5       The heavy chain of clones 2A-H11 and 2A-A9 were recombined with the complete repertoire of light chains derived from the PBL and large (tonsil-derived) scFv repertoires as described earlier for the TGF $\beta$ -1-specific scFv antibody 1-B2. Both repertoires  
10 generated from the recombination with the PBL light chain repertoire were approximately  $1 \times 10^5$ , those generated from the recombination with the tonsil light chain repertoire were approximately  $1 \times 10^6$ .

15   b. Selection of Light Chain Shuffle Repertoires

      The light chain-shuffle repertoires were selected for TGF $\beta$ -2-specific antibodies using biotinylated TGF $\beta$ -2, as described earlier for the selection of the TGF $\beta$ -1 light chain shuffle repertoires. For all of  
20 the first and second round selections, a concentration of 100 nM biotinylated TGF $\beta$ -2 was used. For the third round selection of the PBL-derived light chain shuffle repertoire, biotinylated TGF $\beta$ -2 was used at concentrations of 100 nM and 1 nM. For the third  
25 round selection of the tonsil-derived light chain shuffle repertoire, biotinylated TGF $\beta$ -2 was used at a concentration of 50 nM.

c. Identification of TGF $\beta$ -2-Specific ScFv Antibodies  
from Light Chain Shuffle Repertoires

ScFv antibodies specific to TGF $\beta$ -2 were identified by both phage and soluble ELISA, and  
5 sequenced, as described earlier. Five new TGF $\beta$ -2-specific scFv antibodies were identified. The sequences are summarised below and the complete sequence of each clone given in Figure 2 (b).

| 10 | <u>CLONE</u> | <u>VL SOURCE</u> | <u>VH GERMLINE</u> | <u>VL ISOTYPE</u> |
|----|--------------|------------------|--------------------|-------------------|
|    | 6-H1         | PBL              | DP50 (2A-H11)      | VKappa            |
|    | 6-A5         | PBL              | DP50 (2A-H11)      | VLambda           |
|    | 6-B1         | PBL              | DP50 (2A-H11)      | VLambda           |
| 15 | 11-E6        | PBL              | DP46 (2A-A9)       | VKappa            |
|    | 14-F12       | Tonsil           | DP46 (2A-A9)       | VLambda           |

d. Specificity determination by ELISA

Clones identified as binding TGF $\beta$ -2 rather than  
20 uncoated well, as described above, were further analysed for fine specificity. Specificity ELISA's were carried out using scFv either displayed on phage or in solution as described above, except that 5 ml of media in 50 ml Falcon tubes were inoculated with each  
25 clone and grown to generate the phage or soluble scFv used in the ELISA. Microtitre plate wells were coated with 50  $\mu$ l of either 0.2  $\mu$ g/ml TGF $\beta$ -1, 0.2  $\mu$ g/ml TGF $\beta$ -2, 10  $\mu$ g/ml bovine serum albumin (BSA) or PBS



(the uncoated well). After preblocking both the phage (or soluble scFv) and the microtitre plates, 50  $\mu$ l blocked phage (or soluble scFv) from each clone was added to a well coated with either TGF $\beta$ -1, TGF $\beta$ -2, BSA or an uncoated well. As above, alkaline phosphatase activity was visualised using either the chromagenic substrate pNPP (Sigma) or the Ampak system (Dako). Clones were considered to be specific for TGF $\beta$ -2 if the ELISA signal generated in the TGF $\beta$ -2 coated well was at least five-fold greater than the signal on either TGF $\beta$ -1, BSA or an uncoated well. Cross-reactivity with unrelated antigens was determined more extensively for anti-TGF $\beta$ 2 antibody in whole antibody format, see example 2. The cross-reactivity of 6B1 IgG4 and 6A5 IgG4 with TGF $\beta$ 1 and TGF $\beta$ 3 (obtained from R&D Systems, Abingdon) is also shown to be very low.

e. Specificity determination by BIAcore™

The antibodies were also shown to be specific for TGF $\beta$ 2 compared to TGF $\beta$ 1 by relative binding to the BIAcore sensor chips coated with the appropriate antigen. TGF $\beta$ 1 and TGF $\beta$ 2 were immobilised by amine coupling to Biosensor CM5 sensor chips (Pharmacia) according to the manufacturers instructions. Single chain Fv fragments (35  $\mu$ l; purified by immobilized metal affinity chromatography) were injected over the immobilized antigen at a flow rate of 5  $\mu$ l/min. The

amount of TGF $\beta$  bound was assessed as the total increase in resonance units (RUs) over this period. For the single chain Fv fragments 6H1, 6A5 and 14F12, these fragments gave a total of 686, 480 and 616 RUs respectively for the TGF $\beta$ 1 coated sensor chip and 77, 71 and 115 RUs respectively for the TGF $\beta$ 2 coated chip.

5. *Building higher affinity anti TGF $\beta$ -1 biological neutralisers*

10

a. Recombining heavy chains derived from high affinity anti- TGF $\beta$ 1 scFv with light chains derived from anti-TGF $\beta$ 1 and anti-TGF $\beta$ 2 scFv showing good properties

Antibodies derived by spiking CDR3 of the scFv antibody 1-B2 (section 2b) bind TGF $\beta$ -1 with high affinity. To improve the chance of obtaining high affinity neutralising antibodies it was decided to chain shuffle VHs derived from high affinity anti-TGF $\beta$ -1 scFv with VLs derived from scFv clones with promising properties and particularly with those capable of neutralising the activity of TGF $\beta$ -2 in vitro.

Heavy chains were amplified by PCR from the repertoire of CDR3 spiked 1-B2 clones after selection on TGF $\beta$ -1 (section 2a.ii) using the primers pUC19reverse and PCR-H-Link (Table 1). Amplification conditions consisted of 30 cycles of 94  $^{\circ}$ C for 1 min, 55  $^{\circ}$ C for 1 min and 72  $^{\circ}$ C for 1min, followed by 10 min

at 72 °C. The PCR product was separated through a 1% agarose-TAE gel, the band representing the amplified VH excised, and eluted from the agarose gel using the GeneClean Kit (Bio 101).

5           Light chains were separately amplified by PCR from each of the anti TGFβ-1 specific neutralisers (7-A3, 10-A6 and 14-A1; section 2a.iii) and each of the anti TGFβ-2 specific neutralisers (6H1, 6A5, 6B1, 11E6 and 14F12; section 4c) using the primers fdtetseq1  
10 and PCR-L-Link (Table 1). The same PCR conditions were used as described for the VH amplification. Each VL PCR product was then separately purified through a 1% agarose-TAE gel as described above. Purified products were finally mixed in approximately equimolar  
15 amounts (as estimated from an analytical agarose gel) to provide a VL 'pool'.

          Approximately 50 ng amplified heavy chains and 50 ng of amplified pooled light chains were combined and precipitated with sodium acetate and ethanol using 25  
20 µg glycogen as a carrier. The precipitated DNA was pelleted by centrifugation at 13,000 rpm in a microfuge, air dried and resuspended in 23 µl H<sub>2</sub>O. This was used in an assembly amplification after the addition of reaction buffer, dNTP's to 200 nM and 5  
25 units Taq polymerase. Amplification conditions consisted of 20 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 mins, followed by 10 min at 72 °C. 5 µl of assembly was used as the template in a 50ul

'pull-through' amplification with the primers fdtetseq and pUC19reverse. Amplification conditions consisted of 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2mins, followed by 10 min at 72 °C.

5       The pull-through amplification product was separated through 1% agarose-TAE and the band representing the pull-through VH-VL excised and eluted using the Geneclean Kit. This was digested with the restriction endonucleases Sfi I and Not I (NEB) and  
10       ligated into the phagemid vector pCantab 6 (McCafferty et al. 1994 supra), previously digested with Sfi I and Not I, using the Amersham ligation system. The ligation product was used to transform electrocompetent TG1 cells, plated out on 2YTAG plates  
15       and incubated overnight at 30 °C. A repertoire of approximately  $3 \times 10^6$  individual clones was generated.

b. Selection of chain shuffled repertoire

      The chain shuffled repertoire was selected by a  
20       single round of panning on TGFβ-1 (1ug/ml), as previously described (section 1b).

c. Identification of TGFβ-1 specific scFv antibodies

      ScFv antibodies specific to TGFβ-1 were  
25       identified by phage ELISA and sequenced as described earlier (sections 1d.i and 1e). New TGFβ-1 specific scFv antibodies were identified. Five new high affinity clones were isolated - CS32 which comprises

31G9 VH and 7A3 VL; CS39 which comprises 31G9 VH and 6H1 VL; CS37 which comprises 31G9 VH Figure 1(a) (iii) and 11E6 VL with an Ile for Val substitution at residue 2 (VL sequence given in Figure 14); CS35 which  
5 comprises 31G9 heavy chain with substitutions of Glu for Gln at residue 1, Gln for Glu at residue 5 and 14F12 VL; and CS38 which comprises 31G9 VH with substitutions of Thr for Gln at residue 3, Glu for Gln at residue 5, Leu for Phe at residue 27, Ile for Asn  
10 at residue 56 and Arg for Gln at residue 105 and 6A5 VL.

d. Off-rate determination for single chain Fv fragments binding to TGF $\beta$ 1 and TGF $\beta$ 2

15       The off-rates for binding to TGF $\beta$ 1 or TGF $\beta$ 2 of the single chain Fv fragments described in this example were determined as described by Karlsson et al (R. Karlsson et al, J. Immunol. Methods 145, 229-240, 1991). The results obtained are shown in Table 2,  
20 together with dissociation constants for those which have been determined. These results indicate that high affinity antibodies have been isolated.

6. Identification and Characterisation of an Antibody  
25 which Cross-reacts with both Human TGF $\beta$ -1 and TGF $\beta$ -2 but not TGF $\beta$ -3 by Selection of a Large ScFv Repertoire

a. Panning of the Library and Identification of

### Binders

The large scFv library (described earlier) was induced, phagemid particles rescued and panned as described earlier with the following modifications.

- 5 For the first round of panning,  $10^{12}$  tu library phage in 0.5 ml PBS were used (rather than the standard 2 ml), for the second round,  $3.5 \times 10^9$  phage in 0.5 ml PBS were used. The immuno tube was coated with 10  $\mu$ g TGF $\beta$ -2 in 0.5 ml PBS for both the first and second
- 10 round of selection. Individual colonies from the second selection were screened by ELISA using 0.2  $\mu$ g/ml TGF $\beta$ -1. Clones binding TGF $\beta$ -1 were further screened on TGF $\beta$ -2, TGF $\beta$ -3, BSA and PBS. Clones were considered to be specific for both TGF $\beta$ -1 and TGF $\beta$ -2
- 15 if the ELISA signal generated in the TGF $\beta$ -1 and the TGF $\beta$ -2 coated wells were both at least five-fold greater than the signal on TGF $\beta$ -3, BSA and an uncoated well.

20 c. Identification of a TGF $\beta$ -1/TGF $\beta$ -2 Cross-reactive ScFv Antibody

- A single scFv antibody specific for both TGF $\beta$ -1 and TGF $\beta$ -2 was identified by both phage and soluble ELISA, and sequenced, as described earlier. The
- 25 complete sequence of the VL domain of the antibody gene VT37 is given in figure 4. The dissociation constant of this single chain Fv antibody was estimated by analysis using BIAcore™ to be 4nM for

TGFβ1 and 7nM for TGFβ2. Cross-reactivity for TGFβ3 was also determined. Purified VT37scFv at 8.3μg/ml was passed over BIAcore™ sensor chips coated with TGFβ1 (500RUs coated); TGFβ2 (450RUs coated) or TGFβ3 (5500RUs coated). The relative response for VT37 scFv binding was: TGFβ1 - 391RU bound; TGFβ2 - 261RU bound or TGFβ3 - 24RU bound. Thus this antibody binds strongly to TGFβ1 and TGFβ2 but binding to TGF β 3 is not detectable above background.

10

#### EXAMPLE 2

##### *Construction of Cell Lines Expressing Whole Antibodies*

For the construction of cell lines expressing IgG4 antibodies, variable domains were cloned into  
15 vectors expressing the human gamma 4 constant region for the VH domains or the human kappa or lambda constant regions for the VL domains.

To construct the whole antibody, 27C1/10A6 IgG4 (specific for TGFβ<sub>1</sub>), 27C1 VH DNA was prepared from  
20 the clone isolated above, in example 1. The VH gene was amplified by PCR using the oligonucleotides VH3BackSfiEu and VHJH6ForBam (Table 1) with cycles of 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C. Following digestion with SfiI and BamHI, the VH gene  
25 was cloned into the vector vhcassette2 (Figure 5) digested with SfiI and BamHI. Ligated DNA was transformed into E. coli TG1. Ampicillin resistant colonies were obtained and those containing the

correct insert identified by DNA sequencing.

Plasmid DNA from these colonies was prepared and the DNA digested with HindIII and BamHI. The HindIII-BamHI restriction fragment was ligated into  
5 the human IgG4 heavy chain expression vector pG4D100 (Figure 6), which had been digested with HindIII and BamHI and the DNA transfected into *E. coli* TG1 by electroporation. The sequence of the VH gene insert was again verified by DNA sequencing.

10 For the light chain, the VL gene of 10A6, isolated in example 1, was first mutagenized to remove its internal BamHI site using site directed mutagenesis (Amersham RPN1523) with the oligonucleotide DeltaBamHI (Table 1). The resulting  
15 VLDBamHI gene was amplified by PCR using the oligonucleotides Vλ3/4BackEuApa and HuJλ2-3ForEuBam (Table 1). Following digestion of the amplified insert with ApaI and BamHI, the VL gene was cloned into the vector vlcassetteCAT1 (Figure 7) digested with ApaI  
20 and BamHI. Ligated DNA was transformed into *E. coli* TG1. Ampicillin resistant colonies were obtained and those containing the correct insert were identified by DNA sequencing.

Plasmid DNA from these colonies was prepared and  
25 the DNA digested with Hind III and BamHI. The HindIII-BamHI restriction fragment containing the leader sequence and the VL domain was ligated into the human lambda light chain expression vector, pLN10



(Figure 8), which had been digested with HindIII and BamHI. Following electroporation, transformants in *E. coli* were checked by DNA sequencing.

Plasmid DNA was prepared from the pG4D100-27C1 clone and the pLN10-10A6 clone. This DNA was then co-transfected into DUKXB11 Chinese Hamster Ovary (CHO) cells by electroporation (290V; 960 $\mu$ F). The cells were then grown for 2 days in non-selective medium (alpha-MEM plus nucleosides). Cells were then transferred to a selective medium (alpha-MEM plus 1mg/ml G418 without nucleosides) and grown in 96 well plates. Colonies were then transferred to 24 well plates and samples assayed by sandwich ELISA for assembled human IgG4 antibody and by binding to TGF $\beta$ 1 in ELISA (as in example 1). For the sandwich ELISA, goat anti-human IgG coated on to the ELISA plate and captured human IgG4 detected using goat antihuman lambda light chain alkaline phosphatase conjugate. High expressing cell lines were then derived by amplification of the inserted genes using selection in the presence of methotrexate (R.J. Kaufman Methods Enzymol. 185 537-566, 1990).

The whole antibody 6H1 IgG4 (specific for TGF $\beta$ 2) was constructed in a similar way to the above construction of 27C1/10A6 IgG4. The 6H1 VH gene (example 2) was cloned into pG4D100 as for 27C1 above except that PCR amplification was performed with the oligonucleotides VH3BackSfiEu and VHJH1-2FORBam. The

6H1 VL gene (example 2) was subcloned into vlcassetteCAT1 as above except that PCR amplification was performed with the oligonucleotides Vk2BackEuApa and HuJk3FOREuBam. However, since the 6H1 VL is a kappa light chain the HindIII-BamHI fragment was subcloned into the human kappa light chain expression vector pKN100 (Figure 9) which had been digested with HindIII and BamHI. High expressing cell lines were then isolated as described above. Clones expressing antibody were identified from culture plates by sandwich ELISA for assembled human IgG4 antibody (detected using goat anti-human kappa light chain conjugate and by binding to TGF $\beta$ 2 in ELISA (as in example 2)).

15 To construct the whole antibodies 6A5 IgG4 and 6B1 IgG4, the same 6H1 VH construct in pG4D100 was used as for 6H1IgG4 since these antibodies all have the same VH gene. The 6B1 and 6A5 genes were each subcloned into vlcassetteCAT1 as above for the 10A6 light chain except that PCR amplification was performed with the nucleotides V $\lambda$ 3backEuApa and HuJ $\lambda$ 2-3ForEuBam. The HindIII-BamHI restriction fragment was then subcloned into pLN10 as above. Clones expressing antibody were identified from culture plates by sandwich ELISA for assembled human IgG4 antibody (detected using goat anti-human kappa light chain conjugate and by binding to TGF $\beta$ 2 in ELISA (as in example 2)).

25

*Properties of whole antibody constructs*Purification of whole antibodies

Serum-free supernatant from CHO cells producing

5 the relevant IgG was clarified by centrifugation at 8000 rpm (Beckman JS2-21) prior to purification. The supernatant was applied to a HiTrap Protein A Sepharose prepacked affinity column from Pharmacia, either 1 or 5ml size, with binding capacities of 25 or

10 120 mg respectively. Each IgG had a dedicated column to avoid any potential carry over of material from one purification to another. The column was equilibrated to phosphate buffered saline (PBS) with ten column volumes of 1xPBS prior to applying the supernatant.

15 When all the supernatant had been applied to the column at a flow rate of 2-4 ml/minute, again, depending on the column size, the column was washed with ten column volumes of 1xPBS to remove any non-specifically bound material. Elution of the bound

20 protein was achieved using 0.1M sodium acetate, adjusted to pH 3.3 with glacial acetic acid. The eluted material was collected in 8 fractions of 1.5 ml volume, and the amount of protein determined by measuring the absorbance at 280nm, and multiplying

25 this value by 0.7 to get a value in mg/ml. This was then neutralised with 0.5ml of 1M Tris.HCl pH 9.0 per 1.5ml fraction, and the protein-containing fractions pooled and dialysed against 1x PBS to buffer exchange

the IgG. The column was returned to neutral pH by running ten column volumes of 1xPBS through, and was stored in 20% ethanol as a preservative until required again.

5        A sample was then run on 10-15% SDS-PAGE (Phast system, Pharmacia) and silver stained. this allowed an assessment of the purity of the IgG preparation. This was usually found to be about 80-90%, with only a couple of other bands prominent on the stained gel.

10

#### Binding specificity by ELISA

The IgG4 antibodies 6B1 and 6A5 were shown to bind TGF $\beta$ 2 with very low cross-reactivity to TGF $\beta$ 1 and TGF $\beta$ 3 and no detectable cross-reactivity with a range  
15 of non-specific antigens: interleukin-1; human lymphotoxin (TNF $\beta$ ); human insulin; human serum albumin; single stranded DNA; oxazolone-bovine serum albumin; keyhole limpet haemocyanin; chicken egg white trypsin inhibitor; chymotrypsinogen; cytochrome c;  
20 glyceraldehyde phosphate dehydrogenase; ovalbumin; hen egg lysozyme; bovine serum albumin and tumour necrosis factor  $\alpha$  - (TNF $\alpha$ ) (Figure 13(a) and (b)). Likewise the antibodies 6B1, 6A5 and 6H1 IgG4 bound strongly to TGF $\beta$ 2 coated on a BIAcore™ sensor chip but not  
25 significantly to TGF $\beta$ 1 or TGF $\beta$ 3 coated chips.

#### Binding properties of whole antibodies by BIAcore™

The affinity constants of the above antibodies

were determined by BIACore™, using the method of Karlsson et al. J. Immunol. Methods 145, 299-240, 1991 (supra) and found to be approximately 5nM for 27C1/10A6 IgG4 for TGFβ1 and 2nM for 6H1 IgG4 for TGFβ2. The antibody 27C1/10A6 IgG4 also shows some cross-reactivity with TGFβ2 coated onto Biosensor chips but the dissociation constant is approximately 10 fold or more higher for TGFβ2 compared to TGFβ1. There was no significant cross-reactivity with lysozyme coated onto a BIACore™ sensor chip.

Neutralisation and inhibition of radioreceptor binding by IgG4 antibodies to TGFβ1 and TGFβ 2 is described in examples 3 and 4.

### EXAMPLE 3

#### *Neutralisation by Antibodies of the Inhibitory Effect of TGF β1 and TGF β2 on Cell Proliferation*

The neutralising activity of the antibodies described in examples 1 and 2 were tested in a modification of a bioassay for TGF β as described by Randall et al (1993) J. Immunol Methods 164, 61-67. This assay is based on the ability of TGF β<sub>1</sub> and TGF β<sub>2</sub> to inhibit the interleukin-5 induced proliferation of the erythroleukaemia cell line, TF1 and being able to reverse this inhibition with specific TGF β antibodies.

#### *Method*

Cells and maintenance

The human erythroleukaemia cell line TF1 was grown in RPMI 1640 medium supplemented with 5% foetal calf serum, penicillin/streptomycin and 2ng/ml

- 5 rhGM-CSF in a humidified incubator containing 5% CO<sub>2</sub> at 37°C. Cultures were passaged when they reached a density of  $2 \times 10^5$ /ml and diluted to a density of  $5 \times 10^5$ /ml.

10 Cytokines and Antibodies

rhGM-CSF and rhIL-5 were obtained from R&D systems, rhTGF  $\beta_2$  was obtained AMS Biotechnology. Rabbit anti TGF  $\beta_2$  antibody was from R&D Systems and Mouse anti-TGF  $\beta_{1,2,3}$  was from Genzyme. Other

15 antibodies against TGF  $\beta_2$  were as described in examples 1&2.

Titration of Inhibition of Proliferation by TGF  $\beta_2$ .

- Doubling dilutions of TGF  $\beta_2$  (800pM - 25pM) for
- 20 the construction of a dose response curve were prepared on a sterile microtitre plate in 100 $\mu$ l of RPMI 1640 medium containing 5% foetal calf serum and antibiotics. All dilutions were performed at least in quadruplicate. Additional wells containing 100 $\mu$ l of
- 25 the above medium for reagent and cells only controls were also included.

TF1 cells were washed twice in serum free RPMI 1640 medium and resuspended in RPMI 1640 medium

supplemented with 5% foetal calf serum, 100U/ml penicillin and 100µg/ml streptomycin and 4ng/ml rhIL-5 at a density of  $2.5 \times 10^5$ /ml. Aliquots of 100µl were added to the previously prepared dilution series and  
5 the plate incubated for 48hr. in a humidified incubator containing 5% CO<sub>2</sub> at 37°C.

Cell proliferation was measured colourimetrically by addition of 40µl CellTiter96 substrate (Promega), returning the plate to the incubator for a further 4hr  
10 and finally determining the absorbance at 490nm. The percentage inhibition for each concentration of TGF β<sub>2</sub> as compared to cell only wells was then calculated.

Assay for Neutralisation of TGF β<sub>2</sub> Inhibitory Activity  
15 by Anti-TGF β<sub>2</sub> Antibodies

Neutralisation of TGF β<sub>2</sub> was determined by making doubling dilutions in of each purified antibody in 100µl of medium as above. TGF β<sub>2</sub> was added to each antibody dilution to give a final concentration  
20 equivalent to that which gave 50% inhibition in the titration described above. Each dilution was prepared in quadruplicate. Additional wells were prepared for antibody only, cells only and reagent controls. Cell preparation and determination of cell proliferation  
25 was performed as described above.

Results

TGF β<sub>2</sub> was shown to inhibit the proliferation of

TF1 cells by 50% at a concentration of 50pM. This concentration was used for all neutralisation experiments.

These assays showed that TGF  $\beta_2$  activity was neutralised in a dose dependant manner for both scFv fragments (figure 10) and for whole IgG4 antibodies (figure 11). The concentration of antibody which gave 50% inhibition was determined from the graphs and is shown in table 4.

10

#### EXAMPLE 4

##### *Inhibition by Antibodies of TGF $\beta$ Binding to Receptors Measured in A Radioreceptor Assay*

Single chain Fv fragments and whole IgG4 antibodies from different clones were expressed and purified and their ability to inhibit binding of TGF $\beta$  to receptors measured in a radioreceptor assay.

##### *Purification of scFv*

20 ScFvs containing a poly histidine tail are purified by immobilised metal affinity chromatography. The bacterial clone containing the appropriate plasmid is inoculated into 50 ml 2TY medium containing 2% glucose and 100  $\mu$ g/ml ampicillin (2TYAG) and grown overnight at 30°C. The next day the culture is added to 500 ml prewarmed 2TYAG and grown at 30°C for 1 h. The cells are collected by centrifugation and added to 500 ml 2TY containing ampicillin and 1 mM IPTG and



grown at 30°C for 4 h. The cells are then collected by centrifugation and are resuspended in 30 ml ice-cold 50 mM Tris HCl pH 8.0, 20% (w/v) sucrose, 1 mM EDTA. After 15 min end-to-end mixing at 4°C the mixture is centrifuged at 12 k rpm for 15 min at 4°C. The supernatant is removed and to it added ~ 1ml NTA-agarose (Qiagen 30210) and mixed at 4°C for 30 min. The agarose beads are washed extensively with 50 mM sodium phosphate, 300 mM NaCl and loaded into a small column. After further washing with 50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole pH 7.4 scFv is eluted with 50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole pH 7.4. 0.5 ml fractions are collected and the protein containing fractions identified by measuring the A<sub>280nm</sub>. Pooled fractions are concentrated and scFv further purified by gel filtration in PBS on a Superdex 75 column (Pharmacia).

#### *Purification of Whole Antibodies*

Whole IgG4 antibodies were purified as described in Example 2.

#### *Radioreceptor Assay for TGF- $\beta$*

Neutralisation of TGF- $\beta$  activity is measured by the ability of the scFvs and IgGs to inhibit the binding of <sup>125</sup>I labelled TGF- $\beta$  to its receptors on A549 human lung carcinoma cells.

A549 cells (ATCC CCL 185) are grown in high

glucose Dulbecco's modified Eagle's medium (Sigma D-6546) supplemented with 10% foetal calf serum (PAA), 2 mM glutamine (Sigma G-7513), penicillin/streptomycin (Sigma P-0781), MEM non-essential amino acids (Sigma M-7145).

Cells are seeded at  $1-2 \times 10^5$  cells / ml / well into the wells of 24-well cluster plates and grown for 24 h in serum-free DMEM. Cell monolayers are washed twice with serum-free DMEM and 0.5 ml binding medium (DMEM/Hams F12 (Sigma D-6421) containing 0.1% (v/v) BSA added to each well.

Aliquots of  $^{125}\text{I}$ -TGF- $\beta$ 1 or - $\beta$ 2 (70-90 TBq/mmol; Amersham International) at 20 pM are preincubated with antibody in binding medium at room temperature for 1 h. Duplicate samples of 0.5 ml of TGF- $\beta$ /antibody mixtures are then added to the cell monolayers and are incubated at 37°C for 1-2 h. Control wells contain TGF- $\beta$  only. Unbound TGF- $\beta$  is removed by washing 4 times with Hank's balanced salt solution containing 0.1% BSA. Cells are solubilised in 0.8 ml 25 mM Tris HCl pH 7.5, 10 % glycerol, 1 % Triton X-100 at room temperature for 20 min. The contents of each well are removed and  $^{125}\text{I}$  measured in a gamma counter. The potency of each scFv or IgG is measured by the concentration of antibody combining sites necessary to inhibit binding of TGF- $\beta$  by 50% (IC50; Table 5). Thus the IC50 values are below 10nM and in some cases below 1nM indicating very potent antibodies.

*EXAMPLE 5**Prevention of Scar Formation by Antibodies Against TGF  $\beta_1$  and TGF  $\beta_2$  in the Injured Central Nervous System of the Rat*

5            Logan et al (1994) Eur. J. Neuroscience 6, 355-363  
showed in a rat model of CNS injury, the  
ameliorating effect of a neutralising turkey antiserum  
directed against TGF  $\beta_1$  on the deposition of fibrous  
scar tissue and the formation of a limiting glial  
10    membrane that borders the lesion. A study was set up  
to investigate the effects of neutralising engineered  
human antibodies directed against both TGF  $\beta_1$  and TGF  
 $\beta_2$  in the same rat model. The derivation of the  
antibodies used in this study is described in examples  
15    1 and 2.

*Method*Animals and surgery

20           Groups of five female Sprague-Dawley rats (250g)  
were anaesthetised with an i.p. injection. The  
anaesthetised rats had a stereotactically defined  
lesion made into the right occipital cortex (Logan et  
al 1992 Brain Res. 587, P216-227) and the lateral  
25    ventricle was surgically cannulated and exteriorised  
at the same time (Logan et al 1994 supra).

Neutralisation of TGF  $\beta$

Animals were intraventricularly injected daily with 5ul of purified anti TGF  $\beta$  antibodies (Table 3) diluted in a vehicle of artificial cerebrospinal fluid as described by Logan et al 1994 supra. Fourteen days post lesion all animals were perfusion fixed and 7mm polyester wax sections were processed for histochemical evaluation of the lesion site by immunofluorescent staining.

10 Fluorescent immunohistochemistry and image analysis

Morphological changes within the wound site were followed by immunofluorescent staining with antibodies to fibronectin and laminin detected with anti-species FITC conjugates (Logan et al 1994 supra). These changes were semi-quantitatively assessed by image analysis using a Leitz confocal microscope linked to a Biorad MRC500 laser scanning system. Readings were taken at standard positions mid-way along the lesion.

20 Results

Effects of antibodies to TGF  $\beta$  at the site of CNS injury

Quantitation of the specific relative fluorescence for each of the antibodies is shown in figure 12 a and b. Laminin is a measure of the formation of the glial limitans externa along the boundaries of the wound and together with fibronectin

forms a matrix of fibrous tissue within the centre of the wound. Quantitation by image analysis of these two proteins allows the degree of scarring at the wound site to be determined.

5 Compared with the saline control (fig.12 a,b), There is a considerable decrease in fibronectin and laminin immuno-localisation in the wound in the anti-TGF  $\beta$  antibody treated brains. Thus this indicates that these engineered human antibodies  
10 directed against epitopes on TGF  $\beta_1$  & TGF  $\beta_2$  ameliorate the effects of injury to the CNS both separately and together. by preventing the deposition of the cellular matrix proteins fibronectin and laminin within the wound site. Previously Logan et al (1994  
15 supra) had shown the effectiveness of a polyclonal turkey anti-sera directed against TGF  $\beta_1$ . This is the first report of any antibodies directed against TGF  $\beta_2$  having been shown to be effective in this model.

#### 20 EXAMPLE 6

##### *Determination of Binding of 6B1 IgG4 to Active or Latent Form of TGF $\beta_2$*

TGF $\beta_2$  is synthesised and secreted exclusively as a biologically inactive or latent complex (Pircher et  
25 al, (1986) Biochem. Biophys Res. Commun. 158, 30-37). The latent complex consists of TGF $\beta_2$  disulphide linked homodimer non-covalently associated with latency-associated peptide (LAP). Activation of TGF $\beta_2$  occurs

when it is released from its processed precursor.

Active TGF $\beta_2$  is capable of reversibly dissociating and reassociating with the LAP, which results in the turning on and off of its bio-activity respectively.

5        Cultured PC-3 adenocarcinoma cells (Ikeda et al (1987) Biochemistry 26, 2406-2410) have been shown to secrete almost exclusively latent TGF $\beta_2$  providing a convenient source for determination of binding to the active or latent form of TGF $\beta_2$  by the antibody 6B1  
10    IgG4.

#### *Method*

#### Cell Culture

15        PC-3 prostatic adenocarcinoma cells were grown to confluence in supplemented with 10% FBS. The cells were washed 3x with PBS and cells cultured for a further 7 days in serum free Hams F12/DMEM supplemented with  $1.4 \times 10^{-5}$ M tamoxifen (Brown et al,  
20    (1990) Growth Factors 3, 35-43). The medium was removed, clarified by centrifugation and divided into two 15ml aliquots. One aliquot was acidified for 15 min with 5M HCl by adding dropwise until the pH = 3.5 and then neutralised by the similar addition of 5M  
25    NaOH/1M HEPES pH7.4. This procedure activates the latent TGF $\beta_2$  quantitatively.

#### Competition ELISA

Sixteen wells of an ELISA plate were coated overnight with 100µl 200ng/ml TGFβ<sub>2</sub> in PBS at 4°C. The plate was washed 3x with PBS tween and blocked at 37°C with 200µl of 3% Marvel in PBS.

- 5        The following samples were incubated at room temperature for 1 hour.

400µl Hams F12/DMEM (reagent blank)

400µl Hams F12/DMEM plus 4µg 6B1 IgG4 antibody  
(positive control)

- 10       400µl PC 3 acid activated conditioned media plus  
4µg 6B1 IgG4 antibody (active TGFβ<sub>2</sub> sample)

400µl PC 3 untreated conditioned media plus 4µg  
6B1 IgG4 antibody (latent TGFβ<sub>2</sub> sample)

- The ELISA plate was emptied of blocking solution  
15 and 100µl of one of the above solutions added to sensitised wells in quadruplicate and incubated at room temperature for 2 hours. The plate was washed 3x with PBS/Tween and wells refilled with 100µl of goat anti-human IgG γ chain alkaline phosphatase conjugate  
20 diluted 1:5000 in 1% Marvel/PBS. After 1 hour the wells were washed 3x with PBS/Tween and bound antibody was revealed with p-NPP substrate by absorbance at 405 nm.

25    *Results*

The results of this experiment are shown in Figure 23.

This result clearly shows that pre-incubation

with activated TGF $\beta$ 2 inhibits binding of 6B1 to TGF $\beta$ 2 bound onto an ELISA plate, whereas the latent form does not. This proves that 6B1 IgG4 only binds to the active form of TGF $\beta$ 2.

5

#### EXAMPLE 7

*Neutralisation by antibodies directed against TGF $\beta$ 2 of the inhibitory effect of TGF $\beta$  isoforms on cell proliferation*

10       The neutralising activity of 6B1 IgG4, 6H1 IgG4 (purified as in example 2) and a mouse monoclonal antibody (Genzyme; J.R. Dasch et al., supra) was measured for each of the TGF $\beta$  isoforms, TGF $\beta$ 1, TGF $\beta$ 2 and TGF $\beta$ 3 in the TF1 cell proliferation assay  
15       described in Example 3. The concentration of TGF $\beta$  isoform was 100pM in each assay.

As shown in Figure 16, 6B1 IgG4 strongly neutralises TGF $\beta$ 2 with an IC<sub>50</sub> of approximately 2nM (Table 6). This compares to 10nM for the mouse  
20       monoclonal from Genzyme and 12nM for 6H1 IgG4. Neither 6B1 IgG4 nor 6H1 IgG4 significantly neutralise TGF $\beta$ 1 (Fig. 17). However, there is significant neutralisation of TGF $\beta$ 3 by both 6B1 (IC<sub>50</sub> ca. 11nM) and 6H1 IgG4 ca. 20nM; Fig. 18). This is considerably  
25       less than the neutralisation potency of the Genzyme monoclonal (IC<sub>50</sub> ca. 0.1nM).

Both 6B1 IgG4 and 6H1 IgG4 are stronger neutralisers of TGF $\beta$ 2 activity than of TGF $\beta$ 3



activity. The neutralisation of TGF $\beta$ 3 activity is greater than would be predicted from the relative binding of these two isoforms by the antibodies (example 2) and the relative binding in a radioreceptor assay (example 8).

#### EXAMPLE 8

*Inhibition by antibodies directed against TGF $\beta$ 2 of binding of other TGF $\beta$  isoforms to receptors measured in a radioreceptor assay*

The ability of 6B1 IgG4 to inhibit binding of TGF $\beta$  isoforms to receptors was measured in a radioreceptor assay as described in example 4.

6B1 IgG4 inhibited binding of  $^{125}\text{I}$ -TGF $\beta$ 2 with an IC<sub>50</sub> of 0.05nM. There was no significant inhibition of binding of  $^{125}\text{I}$ -TGF $\beta$ 1 whereas for  $^{125}\text{I}$ -TGF $\beta$ 3 6B1 IgG4 inhibited binding with an IC<sub>50</sub> of approximately 4nM (Table 6). This indicates the potency of 6B1 IgG4 in this assay and its selectivity for the neutralisation of TGF $\beta$ 2 activity. Cross-reactivity with TGF $\beta$ 3 in this assay is less than 2%.

Thus 6B1 IgG4 preferentially inhibits the binding of TGF $\beta$ 2 to its receptors compared with binding of TGF $\beta$ 3.

#### EXAMPLE 9

*Assessment of TGF $\beta$ 1 Antibodies for Therapeutic Use*

The antibodies isolated in Example 1 were

assessed for potential therapeutic value by *in vitro* measurements of the ability to inhibit TGF $\beta$ 1 binding to its receptors and *in vitro* binding properties.

In Example 4 (Table 5) CS32 showed the strongest inhibition of the antibodies tested of the binding of  $^{125}\text{I}$ -TGF $\beta$ 1 to receptors on A549 cells. A further comparison was performed between CS32 and further antibodies (CS35, CS37 and CS38) that were isolated as described in the experiment in Example 1, section 5c.

10 This showed that CS37 appeared to be the most potent of these antibodies in this assay with an  $\text{IC}_{50}$  of approximately 8nM, compared with 40nM for CS32. The  $\text{IC}_{50}$  value for CS32 is higher than in the previous assay (Table 5) because the nature of the assay means

15 that the absolute  $\text{IC}_{50}$  value can vary with assay conditions.

The antibodies 1A-E5 and 1AH-6 (Example 1, section 1f) and antibodies derived from them were much less potent than antibodies derived from 1B2 in

20 neutralising TGF $\beta$  activity in this radioreceptor assay.

Thus CS37 was the most potent antibody candidate as assessed by inhibition of binding of  $^{125}\text{I}$ -TGF $\beta$ 1 to its receptor.

25

#### *Assessment of binding to TGF $\beta$ 3 by anti-TGF $\beta$ 1 antibodies*

The antibodies 14A1 and 10A6 (Example 1, section

2 (a) (iii)) were shown to preferentially bind TGF $\beta$ 1 over TGF $\beta$ 2 and TGF $\beta$ 3 using the same specificity ELISA as was described in Example 1, section 1 (d) (iii), except that microtitre plates were coated with 50 $\mu$ l of  
5 either 0.2 $\mu$ g/ml TGF $\beta$ 1; 0.2 $\mu$ g/ml TGF $\beta$ 2; 0.2  $\mu$ g/ml TGF $\beta$ 3; 10 $\mu$ g/ml bovine serum albumin (BSA) or PBS (the uncoated well). The clones were shown to be specific for TGF $\beta$ 1 since the signal generated in the TGF $\beta$ 1 coated well was at least five fold greater than the  
10 signal on TGF $\beta$ 2 and TGF $\beta$ 3.

Antibodies derived from the same 1B2 lineage as these antibodies, such as 27C1/10A6 IgG4 (which contains the same VL as 10A6 and the 27C1 VH was prepared by mutagenesis of CDR3 residues) should have  
15 the same cross-reactivity against TGF $\beta$ 3.

#### EXAMPLE 10

*Construction of a High Expressing Cell Line for 6B1 IgG4 using the Glutamine Synthase Selection System and*  
20 *Assessment in a Neutralisation Assay*

##### Construction of p6H1 VH gamma4

6B1 VH was amplified from 6H1 pG4D100 (Example 2) by PCR using oligonucleotides P16 and P17. This DNA  
25 was joined by PCR with a 158bp DNA fragment from M13VHPCR1 (R. Orlandi et al Proc. Natl. Acad. Sci. USA 86 3833-3837, 1989) containing a signal sequence, splice sites and an intron, using oligonucleotides P10

and P17. The PCR product was cut with HindIII and ApaI and cloned into HindIII-ApaI cut pGamma4 (Lonza Biologics plc). A plasmid with the correct insertion was identified and designated p6H1 VH gamma4 (see 5 Figure 20). The VH gene and flanking regions were sequenced at this stage.

#### Construction of 6B1ΔBam pLN10

The VL gene of 6B1 was amplified from the clone 10 of 6B1 scFv in pCANTAB6 (Example 1) and subcloned into pUC119. The VL gene was then mutated by in vitro mutagenesis to remove an internal BamHI site, modifying the DNA sequence but not the protein sequence. In vitro mutagenesis was performed using 15 the oligonucleotide LamDeltaBamHI (Table 1) using a kit from Amersham International plc. The mutated VL gene was amplified using the primers Vλ3backEuApa and HuJλ2-3ForEuBam and subcloned as an ApaLI-BamHI fragment into the vector vlcassetteCAT1. The VL gene 20 was then cloned as a HindIII-BamHI fragment into the vector pLN10 (Figure 8) to generate the vector 6B1ΔBam pLN10.

#### Construction of p6B1λ

25 The 6B1 Vλ gene was amplified by PCR from p6B1ΔBam pLN10 using oligonucleotides P22 and P26. The Cλ gene was amplified by PCR from pLN10-10A6 (Example 2) using oligonucleotides P25 and P19. The 2 DNAs

were joined by overlapping PCR using the oligonucleotides P22 and P19 and the product cut with BstBI and EcoRI and cloned into BstBI-EcoRI cut pMR15.1 (Lonza Biologics plc). A plasmid with the correct insertion was identified and designated p6B1λ (Figure 21).

Construction of final expression vector p6B1gamma4gs

p6H1 VHgamma4 and p6B1λ were digested with BamHI and NotI, fragments were purified and ligated together. A plasmid of the desired configuration was identified from transformants and designated p6B1gamma4gs (Figure 22).

Transfection of NS0 with p6B1 gamma4gs

Stable transfectants secreting 6B1 IgG4 were selected by introducing into NS0 myeloma cells p6B1 which includes the glutamine synthetase (gs) gene which allows growth in glutamine-free (G-) medium (C.R. Bebbington et al Bio/Technology 10 169-175, 1992). 40μg p6B1 gamma4gs were linearised by digestion with PvuI. The DNA was electroporated into  $1.5 \times 10^7$  NS0 cells. Cells were then added to G+DMEM/10% FCS and 50μl aliquots distributed into 6 x 96-well plates and allowed to recover for 24h. The medium was then made selective by the addition of 150μl G-DMEM/10%FCS. Three weeks later gs<sup>+</sup> transfectants were screened by ELISA for the ability

to secrete human IgG4 $\lambda$  antibody. The highest producers were expanded and further analysed. From this analysis 5D8 was selected as the candidate production cell line. 5D8 was cloned once by limiting  
5 dilution to give the cell line 5D8-2A6.

*Assessment of 6B1 IgG4 derived from cell line 5D8-2A6 in the TF1 neutralisation assay*

6B1 IgG4 was purified from the GS/NS0 cell line  
10 5D8-2A6 grown in serum-free medium as described in Example 2. The 6B1 IgG4 antibody was assayed in the TF1 neutralisation assay as described in Example 3. An IC<sub>50</sub> value of 1.8nM was obtained in this assay. Subsequent assays of preparations of 6B1 IgG4 derived  
15 from the 5D8-2A6 cell line have indicated values of IC<sub>50</sub> in the range of 0.65 to 2nM. These are comparable to the values obtained for 6B1 IgG4 produced from CHO cells (Example 2) and compare favourably with that obtained for 6H1 IgG4 derived from a CHO cell line  
20 (IC<sub>50</sub> of 15nM). The values obtained for the IC<sub>50</sub> for 6B1 IgG4 and 6H1 IgG4 in this example are more reliable than those obtained in Example 3 and are shown in Table 4, because of improvements in the assay and in the expression and purification of the  
25 antibodies. The IC<sub>50</sub> value may however be expected to vary with the precise conditions of the assay.

Thus the 6B1 IgG4 provides potent neutralisation of TGF $\beta$ 2 and is suitable for use as a therapeutic.

*EXAMPLE 11**Determination of the Epitope on TGF $\beta$ 2 for the Antibody 6B1 using a Peptide Phage Display Library*

5           The antibody 6B1 was further characterised by epitope mapping. This was done by using a peptide phage display library to select peptide sequences that bind specifically to 6B1. These peptide sequences were then compared to the amino acid sequence of  
10 TGF $\beta$ 2. Correlation between peptide sequences that bind to 6B1 and matching parts of the TGF $\beta$ 2 amino acid sequence indicate an epitope of TGF $\beta$ 2 to which 6B1 binds. An "epitope" is that part of the surface of an antigen to which a specific antibody binds.

15           In this example, the peptide library used was constructed as described by Fisch et al (I. Fisch et al (1996) Proc. Natl. Acad. Sci USA 93 7761-7766) to give a phage display library of  $1 \times 10^{13}$  independent clones. Phage displaying peptides that bind to the  
20 antibody 6B1 were selected from this library by panning. This was performed as described in Example 1.

          Purified 6B1 IgG4 antibody at  $10\mu\text{g/ml}$  in 4ml of PBS was coated onto a plastic tube (Nunc; maxisorp) by  
25 incubating overnight at  $4^\circ\text{C}$ . After washing and blocking with MPBS (see Example 1) an aliquot of the peptide library containing  $5 \times 10^{13}$  phage in 4ml 3%MPBS was added to the tube and incubated at room

temperature for 1.5 hours. The tube was washed 10 times with PBST(0.1%), then 10 times with PBS. Bound phage particles were eluted from the tube by adding 4ml of 100mM triethylamine and incubating the tube stationary for 10 minutes at room temperature. The eluted phage were then added to a tube containing 2ml 1M-Tris.HCl (pH7.4) and 10ml 2YT broth. The phage were then added to 20ml of logarithmically growing E. coli TG1 cells and grown for 1 hour shaking at 100rpm at 37°C. The infected cells were then plated on 2YT agar medium with 15µg/ml tetracycline in 243mm x 243mm dishes (Nunc). Plates were incubated at 30°C for 18 hours. Colonies were scraped off the plates into 10 ml 2TY broth containing 15% (v/v) glycerol for storage at -70°C.

250µl of cells from the first round of selection was used to inoculate 500ml 2YT broth (containing 15µg/ml tetracycline) in a 2 litre conical flask and grown overnight, at 30°C with shaking at 280rpm. A 2ml aliquot of this culture was then taken and centrifuged to remove all cells. 1ml of this phage supernatant was the used for a second round of selection as described above. The pattern of phage growth and panning was repeated over a third and a fourth round of selection.

Individual colonies from the fourth round of selection were used to inoculate 100µl 2YT broth (containing 15µg/ml tetracycline) into individual



wells of 96 well tissue culture plates and grown overnight with gentle shaking at 100rpm at 30°C. Glycerol was added to a final concentration of 15% (v/v) and these master plates were stored frozen at  
5 -70°C.

These clones were screened for clones that bound specifically to the antibody 6B1 in ELISA. Cells from the master plates were used to inoculate 96 well tissue culture plates containing 100µl 2YT broth  
10 (containing 15µg/ml tetracycline) per well and grown overnight with gentle shaking at 100rpm at 30°C. The plates were then centrifuged at 2000rpm. The 100µl phage supernatants from each well were recovered and each was mixed with 100µl of 4% skimmed milk powder in  
15 2x PBS. 100µl of each of these was then assayed by phage ELISA. Purified 6B1 IgG4 antibody at 10µg/ml in PBS was coated onto flexible microtitre plates by incubating overnight at 4°C. Control plates coated with an irrelevant IgG4 antibody at 10µg/ml were also  
20 prepared. The ELISAs were performed as described in Example 1, and visualised with the chromagenic substrate pNPP (Sigma).

Approximately 20% of all the clones analysed bound to the 6B1 coated plate. None of the clones  
25 analysed bound to ELISA plates coated with the irrelevant antibody. Binding therefore appeared to be specific for the binding site of the antibody 6B1.

Clones which bound 6B1 were analysed by DNA

sequencing as described by Fisch et al. A total of 31 different clones were sequenced. These were analysed for possible matches with the sequence of TGF $\beta$ 2 using Mac vector software. Of these clones, 12 showed poor  
5 matching with the sequence of TGF $\beta$ 2 and 10 had no similarity at all. However, there were 4 different clones (some of which had been selected more than once) which showed a reasonable match to a region of the TGF $\beta$ 2 sequence between amino acid positions 56 to  
10 69. Table 8 shows the amino acid sequence of the exon of each of these clones that appears to be responsible for binding to 6B1.

None of these clones exactly match the sequence of TGF $\beta$ 2 nor is there a single clear consensus  
15 sequence between the peptide clones. Nevertheless, careful examination of the sequences reveals a match with residues 60 to 64 of TGF $\beta$ 2 (Table 8). Lining up four clones with L at position 64 reveals 2 clones with R at position 60, 1 clone with V at position 61,  
20 2 with L at position 62 and 3 with S at position 63. This provides the sequence RVL $\beta$ SL corresponding to residues 60 to 64 which form part of the alpha helix which forms the heel region of TGF $\beta$ 2. An antibody recognising this structure would not be expected to  
25 make contact with every amino acid residue in the helix and so a peptide mimicking this sequence could have considerable sequence variation at positions that correspond to parts of the helix that do not make

contact. The alpha helix recognised is believed to form part of the receptor binding region of TGF $\beta$ 2 (D.L. Griffith et al. (1996) Proc. Natl. Acad. Sci. USA 93 878-883).

5

#### EXAMPLE 12

*Determination by Immunohistochemistry of Binding of 6B1 IgG4 to TGF $\beta$ 2 in Mammalian Tissue and Absence of Cross Reactivity*

10 To detect TGF $\beta$ 2 in formalin-fixed tissue sections that express the cytokine, the tissue section is generally treated with a protease, pronase E. This digestion step unmask the antigen, possibly activating latent TGF  $\beta$ 2 to give active TGF  $\beta$ 2. 6B1  
15 IgG4 detects only the active form of TGF  $\beta$ 2 (Example 6).

Using 6B1 IgG4 and immunohistochemical methods the distribution of TGF  $\beta$ 2 was determined in formalin fixed-paraffin wax embedded rat normal rat kidney, and  
20 experimentally lesioned rat brain tissue, following pronase E digestion.

The reactivity of 6B1 IgG4 in frozen cryostat sections of acetone post-fixed normal human tissue was also ascertained to determine whether there was any  
25 binding to other antigens in these tissues.

#### Method

Rat Tissue

Paraffin embedded rat tissues were de-waxed and rehydrated through an alcohol series. The sections were then treated with 0.1% pronase E for exactly 8 min and then washed in water. TGF  $\beta$ 2 was detected in the sections using 6B1 IgG4 at 500ng/ml following the protocol provided with a Vectastain ABC (avidin-biotin-complex) kit from Vector Laboratories. On kidney sections, bound antibody was located with alkaline phosphatase and peroxidase was used on rat brain tissues.

Human Tissue

The following human tissue samples were used:

Adrenal, Aorta, Blood, Large intestine, Small intestine, Cerebrum, Kidney, Lymph Node, Liver, Lung, Spleen, Pancreas, Skeletal muscle, Cardiac Muscle, Thyroid, Nerve, Skin, Eye.

Cryostat sections and smears were fixed for 15 minutes in acetone before application of 6B1 IgG4 antibody labelled with FITC using Sigma Immunoprobe kit. The labelled antibody was incubated for 18hr at 4°C, then detected using an indirect alkaline phosphatase method (detection with anti-FITC antibody followed with anti-species enzyme conjugated antibody). In instances where endogenous alkaline phosphatase activity could not be suppressed a peroxidase detection method was used. No pronase

digestion was used in this case, therefore this procedure would detect only antigens with which the antibody cross-reacts.

## 5 *Results*

### Rat Tissue

Rat kidneys displayed positive staining in tubules present on both the apical and the basolateral  
10 side, demonstrating the presence of TGF  $\beta$ 2 in the tissues.

Injured rat brain at 5 days post injury showed positive staining of neurones, astrocytes and macrophages which was absent in normal brain. This  
15 indicates that the TGF  $\beta$ 2 is expressed in rat brain following injury.

### Human Tissue

No specific staining of any tissue was observed  
20 using fixed cryostat sections of the tissues listed above. Therefore 6B1 IgG4 does not cross-react with antigens in these tissues and when used therapeutically will bind only active TGF  $\beta$ 2 in tissue sections detected by immunohistochemical methods.

25

### *EXAMPLE 13*

*Kinetic analysis of the binding of 6B1 single chain Fv and 6B1 IgG4 to TGF $\beta$  isoforms*

Surface plasmon resonance (SPR) can be used to examine real-time interactions between an immobilised ligand and an analyte, and derive kinetic constants from this data. This was performed using the BIAcore 5 2000 system (Pharmacia Biosensor) with the antigen immobilised on a surface, and the antibody as analyte.

The system utilises the optical properties of surface plasmon resonance to detect alterations in protein concentration within a dextran matrix.

10 Antigen is covalently bound to the dextran matrix at a set amount, and as solution containing antibody passes over the surface to which this is attached, antibody binds to the antigen, and there is a detectable change in the local protein concentration, and therefore an 15 increase in the SPR signal. When the surface is washed with buffer, antibody dissociates from the antigen and there is then a reduction in the SPR signal, so the rate of association, and dissociation, and the amount of antibody bound to the antigen at a 20 given time can all be measured. The changes in SPR signal are recorded as resonance units (RU), and are displayed with respect to time along the y-axis of a sensorgram.

The density of immobilised ligand on the surface 25 of a BIAcore chip is important when deriving kinetic data from the sensorgrams generated. It needs to be quite low, so that only a small amount of analyte antibody is needed for saturation of the chip surface.

For simplicity, the density of a chip surface is quoted in RU's, and an ideal amount for a ligand such as TGF $\beta$ 2 or TGF $\beta$ 3 (25kDa) is 400-600 RU's relative to the baseline set during the immobilisation of the  
5 ligand to the surface. The actual amount of TGF $\beta$  that has to be added to get the correct density has to be determined by investigation, but is reproducible once the correct concentration has been found.

Immobilisation of the ligand to the dextran  
10 matrix of the chip surface is facilitated via amine groups, on lysine side chains in the protein, and carboxyl groups in the dextran matrix. The carboxyl groups in the dextran are activated with N-hydroxysuccinimide (NHS) and N-ethyl-N'-(3-  
15 diethylaminopropyl) carbodiimide (EDC) the antigen in acidic solution is then bound to the surface, and finally any unreacted carboxyl groups are blocked with ethanolamine.

The immobilisation of ligand is automated by the  
20 BIACore 2000 machine, and all steps are carried out in the autosampler or in the flowcell, on the dextran surface of the chip. The buffer used throughout the immobilisation procedure, and the analysis of samples is Hepes -buffered saline (HBS) with a surfactant  
25 (Pharmacia Biosensor). The chips (Pharmacia, CM5), have dextran coating on a thin layer of gold. NHS at 100mM and EDC at 400mM are mixed by the autosampler, and then a fixed volume is injected over the flowcell

surface. This is followed by an injection of antigen in a suitable buffer. In the case of TGF $\beta$ , a surface of the correct density was given by using 25-30 $\mu$ g/ml solution of TGF $\beta$ 2 (AMS) OR TGF $\beta$ 3 (R & D systems) in 10mM acetate. After injection of the ligand, the chip is blocked using 1M ethanolamine. The total amount of TGF $\beta$  bound was assessed from the total increase in resonance units over this period.

To determine the kinetic parameters, a series of dilutions of the antibody samples was made in HBS from about 500 $\mu$ g/ml down to less than 1  $\mu$ g/ml, usually through doubling dilutions. After the antibody has been injected over the antigen surface, the surface is washed with HBS, then regenerated by stripping off the bound antibody with a pulse of 100mM HCl. At the higher concentrations of antibody the antigen on the chip surface is saturated, and the off rate is determined on washing with buffer in the dissociation phase. For determination of the on-rate, lower concentrations of antibody are used, giving a linear binding phase in the sensorgram, allowing  $k_{on}$  determination.

The set of dilutions were repeated on a separate preparation of the same antibody.

To manipulate the sensorgrams to obtain kinetic constants  $k_{on}$  and  $k_{off}$ , the BIAevaluation software package is used. For each binding curve used in the calculations, care was taken that the conditions were



appropriate for the determination of kinetic constants.

6B1 IgG4 was purified from the GS/NS0 cell line of Example 10 as in Example 2. 6B1 single chain Fv was expressed intracellularly in *E. coli*, refolded in vitro (using the methodology of WO94/18227), and purified to give a homogeneous product. The values of  $k_{on}$  and  $k_{off}$  were determined for 6B1 IgG4 for binding to both TGF $\beta$ 2 and TGF $\beta$ 3, and for the single-chain Fv 6B1 for binding to TGF $\beta$ 2. The dissociation constant was calculated by dividing  $k_{off}$  by  $k_{on}$ . The values for these kinetic parameters are shown in Table 7.

Thus, 6B1 scFv and 6B1 IgG4 show very low dissociation constants of 2.3nM and 0.89nM respectively for TGF $\beta$ 2, and there is 9% cross-reactivity with TGF $\beta$ 3 (as judged by the ratio of dissociation constants of 6B1 IgG4 for TGF $\beta$ 3 and TGF $\beta$ 2). For comparison, in earlier studies, where the standard errors were greater and the values less precise, the  $K_d$  values for TGF $\beta$ 2 were determined to be 0.7nM for 6A5 scFv (Table 2) and 2nM for 6H1 IgG4 (Example 2). The  $K_d$  values for all the antibodies directed against TGF $\beta$ 2 which share the same 6H1 VH domain are low and below 10nM.

25

#### EXAMPLE 14

*Binding of a Peptide Corresponding to Residues 56 to 69 of TGF $\beta$ 2 to 6B1 IgG4*

A peptide was synthesised corresponding to the amino acids of TGF $\beta$ 2 surrounding the residues RVL $\beta$ SL, the epitope identified from the selection of phage from the peptide display library (Example 11).

5           The 17-mer peptide CGG-TQHSRVLSLYNTIN (TGF $\beta$ 2<sub>56-69</sub>; synthesised by Cambridge Research Biochemicals) contains residues 56 to 69 of TGF $\beta$ 2 with RVL $\beta$ SL (residues 60 to 64) at its centre. The CGG N-terminal extension is a spacer with a cysteine residue to  
10 facilitate coupling of the peptide to carrier proteins. The peptide corresponding to residues 56 to 69 from TGF $\beta$ 1 (TGF $\beta$ 1<sub>56-69</sub>; CGG-TQYSKVL $\beta$ SLYNQHN) was also synthesised. As a control, irrelevant peptide GPEASRPPKLHPG was used.

15           Two approaches were used to confirm that the epitope on TGF $\beta$ 2 for 6B1 IgG4 comprised the amino acids RVL $\beta$ SL.

- (i) Assessment of the ability of 6B1 IgG4 to bind to TGF $\beta$ 2<sub>56-69</sub> and TGF $\beta$ 1<sub>56-69</sub> coupled to BSA by ELISA  
20 (ii) Assessment of the ability of peptides to bind to 6B1 IgG4 coated onto a BIACore sensor chip.

(i) Assessment of the ability of 6B1 IgG4 to bind to TGF $\beta$ 2<sub>56-69</sub> and TGF $\beta$ 1<sub>56-69</sub> coupled to BSA by ELISA

25           The binding of 6B1 IgG4 to synthetic peptides TGF $\beta$ 1<sub>56-69</sub> and TGF $\beta$ 2<sub>56-69</sub> conjugated to BSA was assessed in an ELISA assay. This was compared with the binding of a control antibody 2G6 IgG4 which is an engineered

antibody with a heavy chain containing a VH from an antibody directed against the hapten NIP combined with a light chain containing a VL from an antibody directed against lysozyme.

5

#### Method

Two mg of each of the peptides TGF $\beta$ 1<sub>56-69</sub> and TGF $\beta$ 2<sub>56-69</sub> were conjugated to BSA using an Imject Activated Immunogen Conjugation kit (Pierce).

10 An immunosorp microtitre plate (Nunc) was coated overnight with 10ug/ml of the conjugated peptides in PBS (rows A-D TGF $\beta$ 1<sub>56-69</sub>, rows E-F TGF $\beta$ 2<sub>56-69</sub>) at 100 $\mu$ l/well. The wells were washed 3x with PBS-tween and the following additions made: Column 1 -100 $\mu$ l PBS  
15 in each well as reagent control; Column 2, rows A,B,E and F 200 $\mu$ l of 6B1 IgG4 10 $\mu$ g/ml; Column 2, rows C,D,G and H 200 $\mu$ l of 2G6 IgG4 10 $\mu$ g/ml.

100 $\mu$ l of PBS was put into all the remaining wells. To produce doubling dilutions of the  
20 antibodies, 100 $\mu$ l was removed from each well in column 2 and placed into the next well in column 3. The sample was mixed and 100 $\mu$ l removed and added to the next well in column 4. This procedure was repeated along the plate with the last 100 $\mu$ l being discarded.  
25 The plate was then incubated at 4°C for 18hr.

After 3x washes with PBS-tween the wells were refilled with 100ul of an alkaline phosphatase conjugate of goat F(ab')<sub>2</sub> fragment specific for the

human IgG gamma chain diluted 1:1000 in PBS and incubated for a further 1hr. After 3x further washes with PBS-tween bound antibody was revealed with p-NPP substrate for 20min.

5

### Results

6B1 IgG4 was shown to bind to both conjugated peptides (Figure 15) but the ELISA signal obtained with  $\text{TGF}\beta 1_{56-69}$  was much lower than that obtained with  $\text{TGF}\beta 2_{56-69}$  at an equivalent concentration of 6B1 IgG4. An approximately 8 to 10 times higher concentration of 6B1 IgG4 was required to obtain an equivalent signal with  $\text{TGF}\beta 1_{56-69}$  compared with  $\text{TGF}\beta 2_{56-69}$ . No signal was obtained with the control 2G6 IgG4 antibody with either peptide-BSA conjugate. 6B1 IgG4 therefore strongly binds  $\text{TGF}\beta 2_{56-69}$  and more weakly binds  $\text{TGF}\beta 1_{56-69}$  coupled to BSA.

(ii) Assessment of the ability of peptides to bind to 6B1 IgG4 coated onto a BIAcore sensor chip.

The binding of 6B1 IgG4 to  $\text{TGF}\beta 2_{56-69}$  was confirmed by binding the peptide to 6B1 IgG4 coated on to a BIAcore sensor chip. The determination of binding properties by surface plasmon resonance using the Pharmacia BIAcore 2000 was described in Example 13. The method of creating a BIAcore sensor chip coated with 6B1 IgG4 was as for the method for coupling with  $\text{TGF}\beta$ , described in Example 13, except

that 6B1 IgG4 was coupled at 5µg/ml in 10mM acetate buffer, pH3.5. A surface of 5000RU was generated using 25µl of 6B1 IgG4.

Twenty µl of the the peptides were applied to the 6B1 surface at 1mg/ml with regeneration of the surface using an acid pulse to remove bound peptide between samples. The amount of binding was assessed by setting a baseline response of absolute RU prior to injection, and then subtracting this from the value at 20 seconds after the injection was complete to give a relative response in RU. This is taken to be the amount of binding to the 6B1 surface.

The binding obtained is shown in Table 9. There was a very low level of binding of the irrelevant peptide. TGFβ1<sub>56-69</sub> appeared to bind specifically at a low level to 6B1 IgG4. However, the TGFβ2<sub>56-69</sub> peptide bound to 6B1 IgG4 specifically and very much more strongly.

The low level of binding of 6B1 IgG4 to the TGFβ1 peptide in the ELISA and BIAcore assays is not unexpected given that 10 of the 14 TGFβ amino acids are identical with the TGFβ2 peptide. Nevertheless, 6B1 IgG4 binds the TGFβ2<sub>56-69</sub> peptide very much more strongly than it binds the TGFβ1<sub>56-69</sub> peptide. The level of discrimination between these TGFβ1 and TGFβ2 peptides is very much lower however than is seen for the radioreceptor (Table 6) and neutralisation assays (Table 6 and Figures 16 and 17) with native isoforms.

In these assays, 6B1 IgG4 strongly neutralises TGF $\beta$ 2 but has little effect on TGF $\beta$ 1 biological activity. This greater discrimination presumably reflects the context of the residues of the peptides in the native isoforms.

### Conclusions

These results support the assignment of the epitope of 6B1 IgG4 on TGF $\beta$ 2 to the aminoacids in the region of residues 60 to 64. The peptide used in this example, residues 56 to 69, corresponds to the amino acids of alpha helix H3 (M.P. Schlunegger & M.G. Grutter Nature 358 430-434, 1992). TGF $\beta$ 2 forms a head-to-tail dimer with the alpha helix H3 (also referred to as the heel) of one subunit forming an interface with finger regions (including residues 24 to 37 and residues in the region of amino acids 91 to 95; also referred to as fingers 1 and 2) from the other subunit (S. Daopin et al Proteins: Structure, Function and Genetics 17 176-192, 1993). It has been proposed that the primary structural features which interact with the TGF $\beta$ 2 receptor consist of amino acids at the C-terminal end of the alpha helix H3 from one chain together with residues of fingers 1 and 2 of the other chain (D.L. Griffith et al Proc. Natl. Acad. Sci. USA 93 878-883, 1996). The identification of an epitope for 6B1 IgG4 within the alpha helix H3 of TGF $\beta$ 2 is consistent with 6B1 IgG4 preventing receptor

binding and neutralising the biological activity of TGF $\beta$ 2.

If the epitope for 6B1 IgG4 is three dimensional there may be other non-contiguous epitopes to which the antibody may bind.

There is earlier evidence that antibodies directed against this region of TGF $\beta$ 2 may be specific for TGF $\beta$ 2 and neutralise its activity. Flanders et al (Development 113 183-191 1991) showed that polyclonal antisera could be raised in rabbits against residues 50 to 75 of mature TGF $\beta$ 2 and that these antibodies recognised TGF $\beta$ 2 but not TGF $\beta$ 1 in Western blots. In an earlier paper, K.C. Flanders et al (Biochemistry 27 739-746, 1988) showed that polyclonal antisera raised in rabbits against amino acids 50 to 75 of TGF $\beta$ 1 could neutralise the biological activity of TGF $\beta$ 1. The antibody we have isolated and characterised, 6B1 IgG4, is a human antibody directed against amino acids in this region which neutralises the biological activity of human TGF $\beta$ 2. It is surprising that such a neutralising antibody against TGF $\beta$ 2 can be isolated in humans (where immunisation with a peptide cannot be used for ethical reasons) directly from a phage display antibody repertoire.

Table 1: Oligonucleotide primers used in the identification and characterisation of TGF- $\beta$ 1 antibodies.

| Primer           | Nucleotide sequence 5' to 3'  |
|------------------|---|
| 132 MutVHCDR3    | 5' CGT GGT CCC TTT GCC CCA GAC GTC CAC ACC ACT AGA ATC GTA GCC ACT ATA TTC CCC<br>AGT TCG CGC ACA GTA ATA CAC ACC CGT |
| pUC19-reverse    | 5' AGC GGA TAA CAA TTT CAC ACA GG 3'  |
| fdtet seq        | 5' GTC GTC TTT CCA GAC GTT AGT 3'   |
| PCR-H-Link       | 5' ACC GCC AGA GCC ACC TCC GGC 3'   |
| PCR-L-Link       | 5' GGC GGA GGT GGC TCT GGC GGT 3'   |
| myc seq 10       | 5' CTC TTC TGA GAT GAG TTT TTG 3'   |
| HUJH4-SFor       | 5' TGA GGA GAC GGT GAC CAG GGT TCC 3'   |
| RL1              | 5' G(C/A)A CCC TGG TCA CCG TCT CCT CA GGT GGA GGC GGT TCA GGC GGA GGT GGC AGC<br>GGC GGT GGC GGA TCG 3'               |
| RL2              | 5' GGA CAA TGG TCA CCG TCT CTT CA GGT GGA GGC GGT TCA GGC GGA GGT GGC AGC<br>GGC GGT GGC GGA TCG 3'                   |
| RL3              | 5' GGA CCA CGG TCA CCG TCT CCT CA GGT GGA GGC GGT TCA GGC GGA GGT GGC AGC<br>GGC GGT GGC GGA TCG 3'                   |
| VH1b/7a back Sfi | 5'-GTC CTC CCA ACT GCG GCC CAG CCG GCC ATG GCC CAG (AG)TG CAG CTG CA(AG) TCT GG-3'                                    |
| WH1c back Sfi    | 5'-GTC CTC CCA ACT GCG GCC CAG CCG GCC ATG GCC (GC)AG GTC CAG CTG GT(AG) CAG TCT GG-3'                                |



123

VH2b back Sfi  
 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG (AG)TC ACC TTG AAG GAG TCT GG-3'  
  
 VH 3b back Sfi  
 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC (GC)AG GTG CAG CTG GTG GAG TCT GG-3'  
  
 VH3c back Sfi  
 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTG CAG CTG GTG GAG (AT)C(TC) GG-3'  
  
 VH4b back Sfi  
 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTA CAG TGG GG-3'  
  
 VH4c back Sfi  
 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG (GC)TG CAG CTG CAG TC(GC) GG-3'  
  
 VH5b back Sfi  
 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GA(AG) GTG CAG CTG GTG CAG TCT GG-3'  
  
 VH 6a back Sfi  
 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTA CAG CTG CAG TCA GG-3'  
  
 VH3BACKSfiEu 5'- AGC TCG GTC CTC GCA ACT GCG GCC CCT GGG GCC CAC AGC GAG GTG CAG CTG GTG  
 GAG TCT GG - 3'  
  
 VHJH6FORBam 5'-CGA GTC ATT CTG CAC TTG GAT CCA CTC ACC TGA GGA GAC GGT GAC CGT GGT CCC - 3'  
  
 DeltaBamHI 5'-GA GAA TCG GTC TGG GAT TCC TGA GGG CCG G-3'  
  
 VL3/4BackEuApa 5'- AGC TCG GTC CTC GCA ACT GGT GTG CAC TCC CAC GTT ATA CTG ACT CAG GAC CC -3'  
  
 HuJλ2-3ForEuBam 5'-G GTC CTC GCA ACT GCG GAT CCA CTC ACC TAG GAC GGT CAG CTT GGT CCC- 3'  
  
 VHJH1-2FORBam 5'-CGA GTC ATT CTG CAC TTG GAT CCA CTC ACC TGA GGA GAC GGT GAC CAG GGT GCC - 3'

Vk2BackEuApa 5'- AGC TCG GTC CTC GCA ACT GGT GTG CAC TCC GAT GTT GTG ATG ACT CAG TCT CC-3'

HuJkFozEuBam 5'-G GTC CTC GCA ACT GCG GAT CCA CTC ACG TTT GAT ATC CAC TTT GGT CCC -3'

Vλ3BackEuApa 5'- AGC TCG GTC CTC GCA ACT GGT GTG CAC TCC TCG TCT GAG CTG ACT CAG GAC CC -3'

LamDeltaBamHI 5'- C CGG CCC TCA GGA ATC CCA GAC CGA TTC TC- 3'

P10 5'-CTA AGC TTA CTG AGC ACA CAG GAC CTC ACC-3'

P16 5'-TTT GGA TAT CTC TCC ACA GGT GTC CAC TCC GAG GTG CAG CTG GTG GAG TCT G-3'

P17 5'-ATG GGC CCT TGG TGG AAG CTG AAG AGA CGG TGA CCA GGG TGC C-3'

P19 5'-TTG AAT TCA GGT GGG GGC ACT TCT CCC TCT ATG AAC ATT CCG TAG GGG CCA CTG TCT TC-3'

P22 5'-TTA ACG ATT TCG AAC GCC ACC ATG GGA TGG AGC TGT ATC ATC CTC-3'

P25 5'-GTC CTA GGT GAG TAG ATC TAT CTG GGA TAA GCA TGC TGT TTT C-3'

P26 5'-GAT CTA CTC ACC TAG GAC GGT CAG CTT GG-3'

Table 2 Properties of single chain Fv fragments for binding to TGFbeta1 or TGFbeta2 determined using BIACore

| <i>Antibody</i> | <i>k<sub>off</sub> (s<sup>-1</sup>)</i> | <i>K<sub>d</sub>(nM)</i> |
|-----------------|---|--------------------------|
| <u>TGFbeta1</u> |   |                          |
| 31G9            | $9.0 \times 10^{-4}$                    | 12                       |
| CS32            | $1.2 \times 10^{-3}$                    |                          |
| CS39            | $1.7 \times 10^{-3}$                    |                          |
| <u>TGFbeta2</u> |   |                          |
| 6A5             | $1.4 \times 10^{-4}$                    | 0.7                      |
| 6B1             | $6.0 \times 10^{-4}$                    |                          |
| 6H1             | $1.1 \times 10^{-3}$                    |                          |
| 14F12           | $2.1 \times 10^{-3}$                    |                          |

Table 3 Daily dose levels for individual animals in each group

| Group | Clone              | Antibody<br>format | Antigen                        | Dose                  |
|-------|--------------------|--------------------|--------------------------------|-----------------------|
| 1     | Saline<br>Control  | -                  | -                              | -                     |
| 2     | 31G9               | scFv               | TGF $\beta_1$                  | 20ng                  |
| 3     | 6A5                | scFv               | TGF $\beta_2$                  | 20ng                  |
| 4     | 27C1/10A6          | IgG4               | TGF $\beta_1$                  | 692ng                 |
| 5     | 6H1                | IgG4               | TGF $\beta_2$                  | 1.76 $\mu$ g          |
| 6     | 31G9<br>+6A5       | scFv's             | TGF $\beta_1$<br>TGF $\beta_2$ | 20ng<br>"             |
| 7     | 27C1/10A6<br>+ 6H1 | IgG4's             | TGF $\beta_1$<br>TGF $\beta_2$ | 692ng<br>1.76 $\mu$ g |

Table 4 I.C.<sub>50</sub> values for antibodies in TF1 assay

| Antibody | scFv<br>(nM) | IgG4<br>(nM) |
|----------|--------------|--------------|
| 6H1      | 1.5          | 100          |
| 6B1      | 15           | 11           |
| 6A5      | 8            | 150          |
| 14F12    | 90           | nd           |

nd = not determined

Table 5 IC<sub>50</sub> values for antibodies measured using a radioreceptor assay.

| Anti-TGF- $\beta$ 1 antibody | IC <sub>50</sub> , nM |
|------------------------------|-----------------------|
|------------------------------|-----------------------|

|          |      |
|----------|------|
| 7A3 scFv | >100 |
|----------|------|

|           |    |
|-----------|----|
| 31G9 scFv | 30 |
|-----------|----|

|           |     |
|-----------|-----|
| CS32 scFv | 4.5 |
|-----------|-----|

|           |     |
|-----------|-----|
| CS39 scFv | ~60 |
|-----------|-----|

|               |   |
|---------------|---|
| 27C1/10A6 IgG | 9 |
|---------------|---|

|           |      |
|-----------|------|
| VT37 scFv | ~100 |
|-----------|------|

| Anti-TGF- $\beta$ 2 antibody | IC <sub>50</sub> , nM |
|------------------------------|-----------------------|
|------------------------------|-----------------------|

|          |     |
|----------|-----|
| 6A5 scFv | 1.5 |
|----------|-----|

|         |    |
|---------|----|
| 6A5 IgG | ~6 |
|---------|----|

|          |     |
|----------|-----|
| 6B1 scFv | 0.3 |
|----------|-----|

|         |     |
|---------|-----|
| 6B1 IgG | 0.6 |
|---------|-----|

|          |      |
|----------|------|
| 6H1 scFv | 0.22 |
|----------|------|

|         |     |
|---------|-----|
| 6H1 IgG | ~10 |
|---------|-----|

|          |     |
|----------|-----|
| 11E6 IgG | 1.6 |
|----------|-----|

|            |   |
|------------|---|
| 14F12 scFv | 3 |
|------------|---|

|           |   |
|-----------|---|
| VT37 scFv | 2 |
|-----------|---|

Table 6 Potency of neutralisation of TGFbeta isoforms

| <i>TF1 cell proliferation assay IC<sub>50</sub> (nM IgG)</i>  |                 |                |
|---|-----------------|----------------|
|   | <u>6B1 IgG4</u> | <u>Genzyme</u> |
| TGFbeta1  | >100            | 1.5            |
| TGFbeta2  | 2               | 10             |
| TGFbeta3  | 11              | 0.1            |
| <i>A549 cell radioreceptor assay IC<sub>50</sub> (nM IgG)</i> |                 |                |
|   | <u>6B1 IgG4</u> | <u>Genzyme</u> |
| TGFbeta1  | >400            | 0.55           |
| TGFbeta2  | 0.05            | 0.5            |
| TGFbeta3  | 4               | 0.03           |

Table 7 Kinetic parameters of 6B1 IgG4 and 6B1 single chain Fv

| antibody format | antigen | k <sub>off</sub> s <sup>-1</sup> | k <sub>on</sub> M <sup>-1</sup> s <sup>-1</sup> | dissociation<br>constant K <sub>d</sub><br>nM |
|-----------------|---------|----------------------------------|---|---|
| 6B1 scFv        | TGFβ2   | 6.68 × 10 <sup>-4</sup>          | 2.87 × 10 <sup>5</sup>                          | 2.32  |
| 6B1 IgG4        | TGFβ2   | 3.36 × 10 <sup>-4</sup>          | 3.84 × 10 <sup>5</sup>                          | 0.89  |
| 6B1 IgG4        | TGFβ3   | 4.5 × 10 <sup>-4</sup>           | 4.5 × 10 <sup>4</sup>                           | 10.0  |



Table 8    Peptide sequences from phage binding to 6B1 IgG4

This table shows the amino acid sequence of 4 phage peptide display clones that show a match with the sequence of TGFbeta2. These clones have been lined up below the relevant part of the sequence of TGFbeta2, which is shown from amino acid positions 56 to 77.

|          |                       |
|----------|-----------------------|
| TGFbeta2 | TQHSRVLSLYNTINPEASAPC |
| Clone 1  | RQLSLQORMH            |
| Clone 2  | DPM DMVLKLC           |
| Clone 3  | WSEFMRQSSL            |
| Clone 3  | VESTSLQFRG            |

| peptide                        | concentration of peptide, $\mu$ M | amount of binding to 6B1 IgG4 surface, RU |
|--------------------------------|-----------------------------------|---|
| TGF $\beta$ 2 <sub>56-69</sub> | 537                               | 1012.8                                    |
| TGF $\beta$ 1 <sub>56-69</sub> | 524                               | 190.7                                     |
| irrelevant peptide             | 745                               | 60.9                                      |

Table 9 Binding of peptides from TGFbeta to 6B1 IgG4 immobilised on a BIACore chip

CLAIMS:

1. A specific binding member comprising a human antibody antigen binding domain specific for human TGF $\beta$  which binds the human TGF $\beta$  isoforms TGF $\beta$ 2, TGF $\beta$ 1, or  
5 TGF $\beta$ 2 and TGF $\beta$ 1, preferentially over TGF $\beta$ 3.
2. A specific binding member according to claim 1 which neutralises TGF $\beta$ 2, TGF $\beta$ 1, or TGF $\beta$ 2 and TGF $\beta$ 1.
3. A specific binding member according to claim 1 or claim 2 wherein said human antibody antigen binding  
10 domain is for the TGF- $\beta$  isoform TGF- $\beta$ 2.
4. A specific binding member according to claim 3 wherein said human antibody antigen binding domain comprises a VH domain which has the amino acid sequence shown in Figure 2(a) (i) or Figure 2(a) (ii).
- 15 5. A specific binding member according to claim 3 or claim 4 wherein said human antibody antigen binding domain comprises a VL domain which has the amino acid sequence shown in any of Figures 2(b) (i) to (v)
6. A specific binding member according to claim 5  
20 wherein said human antibody antigen binding domain comprises a pairing of a VH domain and a VL domain selected from:  
(a) 6H1 VH, of which the amino acid sequence is shown

- in Figure 2(a) (i), and 6B1 VL, of which the amino acid sequence is shown in Figure 2(b) (iii);
- (b) 6H1 VH, of which the amino acid sequence is shown in Figure 2(a) (i), and 6H1, of which the amino acid sequence is shown in Figure 2(b) (i);
- (c) 6H1 VH, of which the amino acid sequence is shown in Figure 2(a) (i), and 6A5 VL, of which the amino acid sequence is shown in Figure 2(b) (ii).

7. A specific binding member according to claim 6 wherein said human antibody antigen binding domain comprises the VH domain 6H1 VH, of which the amino acid sequence is shown in Figure 2(a) (i), and the VL domain 6B1 VL, of which the amino acid sequence is shown in Figure 2(b) (iii).

8. A specific binding member according to claim 3 wherein said human antibody antigen binding domain comprises a complementarity determining region (CDR) with an amino acid sequence identified as a CDR in any of the sequences shown in Figures 19 (i) to (iv).

9. A specific binding member according to claim 8 wherein said human antibody antigen binding domain comprises a VH domain which comprises a CDR3 with a sequence shown as CDR3 in Figure 19 (i).

10. A specific binding member according to claim 3

which competes for binding to TGF- $\beta$ 2 with a specific binding member according to claim 6.

11. A specific binding member according to claim 10 which competes for binding to TGF- $\beta$ 2 with a specific  
5 binding member according to claim 7.

12. A specific binding member according to claim 3 which binds the peptide TQHSRVLSLYNTIN.

13. A specific binding member according to claim 3 which binds the active form of TGF $\beta$ 2 but not the latent  
10 form.

14. A specific binding member according to claim 3 wherein said human antibody antigen binding domain comprises a VH sequence of the DP50 germ line, or a re-arranged form thereof.

15 15. A specific binding member according to claim 1 or claim 2 wherein said human antibody antigen binding domain is for the TGF- $\beta$  isoform TGF- $\beta$ 1.

16. A specific binding member according to claim 15 wherein said human antibody antigen binding domain  
20 comprises a VH domain which has the amino acid sequence shown in any of Figure 1(a) (i), Figure 1(a) (ii) and Figure 1(c) (i).

17. A specific binding member according to claim 15  
or claim 16 wherein said human antibody antigen binding  
domain comprises a VL domain which has the amino acid  
sequence shown in any of Figures 1(b) (i), 1(b) (ii)  
5 and 1(a) (iii).

18. A specific binding member according to claim 17  
wherein said human antibody antigen binding domain  
comprises a pairing of a VH domain and a VL domain  
selected from:

10 (a) 1B2 VH, of which the amino acid sequence is shown  
in Figure 1(a) (i), and 7A3 VL, of which the amino acid  
sequence is shown in Figure 1(b) (i);

(b) 31G9 VH, of which the amino acid sequence is  
shown in Figure 1(a) (ii), and 31G9 VL, of which the  
15 amino acid sequence is shown in Figure 1(a) (iii);

(c) 27C1 VH, of which the amino acid sequence is  
shown in Figure 1(c) (i), and 10A6 VL, of which the  
amino acid sequence is shown in Figure 1(b) (ii).

19. A specific binding member according to claim 18  
20 wherein said human antibody antigen binding domain  
comprises the VH domain 27C1 VH, of which the amino  
acid sequence is shown in Figure 1(c) (i), and the VL  
domain 10A6 VL, of which the amino acid sequence is  
shown in Figure 1(b) (ii).

25 20. A specific binding member according to claim 15

wherein said human antibody antigen binding domain comprises a VH domain which comprises a CDR3 with an amino acid sequence selected from those shown in Figure 3.

5 21. A specific binding member according to claim 20 wherein said CDR3 has the sequence shown for CDR3 of 27C1 VH.

22. A specific binding member according to claim 15 wherein said human antibody antigen binding domain is  
10 comprises the 31G9 VH domain of which the sequence is shown in Figure 1(a) (ii) and the CS37 VL of which the sequence is shown in Figure 14.

23. A specific binding member according to claim 15 which competes for binding to TGF- $\beta$ 1 with a specific  
15 binding member according to claim 18.

24. A specific binding member according to claim 23 which competes for binding to TGF- $\beta$ 1 with a specific binding member according to claim 19.

25. A specific binding member according to claim 15  
20 which competes for binding to TGF $\beta$ 1 with a specific binding member according to claim 22.

26. A specific binding member according to claim 15

which binds the peptide TQYSKVLSLYNQHN.

27. A specific binding member according to claim 1 wherein said human antibody antigen binding domain is for the TGF- $\beta$  isoforms TGF- $\beta$ 1 and TGF- $\beta$ 2.

5 28. A specific binding member according to claim 27 wherein said human antibody antigen binding domain comprise a VL domain with the amino acid sequence shown in Figure 4 and a VH domain with the amino acid sequence shown in Figure 1(a) (ii).

10 29. A specific binding member according to claim 27 which competes for binding to TGF- $\beta$ 1 and for binding to TGF- $\beta$ 2 with a specific binding member according to claim 28.

30. A specific binding member according to any  
15 preceding claim comprising a single-chain Fv antibody molecule.

31. A specific binding member according to any of claims 1 to 29 which comprises one or more amino acids in addition to those forming said human antibody  
20 antigen binding domain.

32. A specific binding member according to claim 31 comprising an antibody constant region.



33. A specific binding member according to claim 32 which comprises a whole antibody.

34. A specific binding member according to claim 32 or 33 wherein said antibody constant region is IgG4  
5 isotype.

35. A method comprising causing or allowing binding of a specific binding member according to any preceding claim to TGF- $\beta$ 1 isoform and/or TGF- $\beta$ 2 isoform of human TGF- $\beta$ .

10 36. A method according to claim 35 wherein binding takes place *in vitro*.

37. A method according to claim 35 wherein binding takes place *in vivo*.

38. A method according to any of claims 35 to 37  
15 wherein said binding of the specific binding member neutralises said isoform or isoforms.

39. Use of a specific binding member according to any of claims 1 to 34 in the manufacture of a medicament for treating an individual to counteract effects of  
20 TGF- $\beta$  which are deleterious to the individual.

40. Use according to claim 39 wherein said effects

are fibrosis promoting effects.

41. Use according to claim 40 wherein said individual has a condition selected from the group consisting of glomerulonephritis, neural scarring, dermal scarring, ocular scarring, lung fibrosis, arterial injury, proliferative retinopathy, retinal detachment, adult respiratory distress syndrome, liver cirrhosis, post myocardial infarction, post angioplasty restenosis, keloid scarring, scleroderma, vascular disorders, cataract, and glaucoma.

42. Use according to claim 41 wherein said condition is neural scarring or glomerulonephritis.

43. Use according to claim 39 wherein said effects contribute to an immune or inflammatory disease condition.

44. Use according to claim 43 wherein said condition is selected from the group consisting of rheumatoid arthritis, macrophage deficiency disease and macrophage pathogen infection.

45. Nucleic acid encoding a specific binding member according to any of claims 1 to 34.

46. Nucleic acid according to claim 45 which is part

of an expression vector.

47. A method which comprises use of nucleic acid according to claim 45 or claim 46 in an expression system for production of a specific binding member  
5 according to any of claims 1 to 29.

48. A host cell containing nucleic acid according to claim 45 or claim 46.

49. A host cell according to claim 48 which is capable of producing said specific binding member under  
10 appropriate culture conditions.

50. A method of producing a specific binding member according to any of claims 1 to 34 comprising culturing a host cell according to claim 49 under appropriate conditions for production of said specific binding  
15 member.

51. A method according to claim 50 wherein following said production said specific binding member is isolated from the cell culture.

52. A method according to claim 51 wherein following  
20 said isolation the specific binding member is used in formulation of a composition comprising at least one additional component.

53. A method according to claim 52 wherein said composition is a pharmaceutical composition comprising a pharmaceutically acceptable excipient.

54. A pharmaceutical composition comprising a  
5 specific binding member according to any of claims 1 to 34 and a pharmaceutically acceptable excipient.

55. A method of treatment of a condition in which effects of TGF- $\beta$  are deleterious to an individual, the method comprising administration of a pharmaceutical  
10 composition according to claim 54 to the individual.

56. A method according to claim 50 wherein said effects are fibrosis promoting effects.

57. A method according to claim 56 wherein said individual has a condition selected from the group  
15 consisting of glomerulonephritis, neural scarring, dermal scarring, ocular scarring, lung fibrosis, arterial injury, proliferative retinopathy, retinal detachment, adult respiratory distress syndrome, liver cirrhosis, post myocardial infarction, post angioplasty  
20 restenosis, keloid scarring, scleroderma, vascular disorders, cataract, and glaucoma.

58. A method according to claim 57 wherein said condition is neural scarring or glomerulonephritis.

59. A method according to claim 55 wherein said effects contribute to an immune or inflammatory disease condition.

60. A method according to claim 59 wherein said condition is selected from the group consisting of rheumatoid arthritis, macrophage deficiency disease and macrophage pathogen infection.

Figure 1(a)(i)

```

      10      20      30      40
CAG GTG CAA CTG GTG GAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AGG
Q V Q L V E S G G G V V Q P G R>

      50      60      70      80      90
TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT AGC TAT
S L R L S C A A S G F T F S S Y>

      100      110      120      130      140
GGC ATG CAC TGG GTC CGC CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG
G M H W V V R Q A P G K G L E W V>

      150      160      170      180      190
GCA GTT ATA TCA TAT GAT GGA AGT AAT AAA TAC TAT GCA GAC TCC GTG
A V I S Y D G S N K Y Y A D S V>

      200      210      220      230      240
AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT
K G R F T I S R D N S K N T L Y>

      250      260      270      280
CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT
L Q M N S L R A E D T A V Y Y C>

      290      300      310      320      330
GCG AAA ACT GGG GAA TAT AGT GGC TAC GAT TCT AGT GGT GTG GAC GTC
A K T G E Y S G Y D S S G V D V>

      340      350      360
TGG GGC AAA GGG ACC ACG GTC ACC GTC TCC TCA
W G K G T T V T V S S

```

Figure 1(a)(ii)

```

10      20      30      40
CAG GTG CAG CTG GTG CAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AGG
Q  V  Q  L  V  Q  S  G  G  G  V  V  Q  P  G  R>

50      60      70      80      90
TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT AGC TAT
S  L  R  L  S  C  A  A  S  G  F  T  F  S  S  Y>

100     110     120     130     140
GGC ATG CAC TGG GTC CGC CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG
G  M  H  W  V  R  Q  A  P  G  K  G  L  E  W  V>

150     160     170     180     190
GCA GTT ATA TCA TAT GAT GGA AGT ATT AAA TAC TAT GCA GAC TCC GTG
A  V  I  S  Y  D  G  S  I  K  Y  Y  A  D  S  V>

200     210     220     230     240
AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT
K  G  R  F  T  I  S  R  D  N  S  K  N  T  L  Y>

250     260     270     280
CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT
L  Q  M  N  S  L  R  A  E  D  T  A  V  Y  Y  C>

290     300     310     320     330
GCG CGA ACT GGT GAA TAT AGT GGC TAC GAT ACG AGT GGT GTG GAG CTC
A  R  T  G  E  Y  S  G  Y  D  T  S  G  V  E  L>

340     350     360
TGG GGG CAA GGG ACC ACG GTC ACC GTC TCC TCA
W  G  Q  G  T  T  V  T  V  S  S

```

Figure 1(a) (iii)

```

      10      20      30      40
GAC ATC GTG ATG ACC CAG TCT CCT TCC ACC CTG TCT GCA TCT GTA GGA
D I V M T Q S P S T L S A S V G>

      50      60      70      80      90
GAC AGA GTC ACC ATC ACT TGC CGG GCC AGT CAG GGT ATT AGT AGC TGG
D R V T I T C R A S Q G I S S W>

      100     110     120     130     140
TTG GCC TGG TAT CAG CAG AAA CCA GGG AGA GCC CCT AAG GTC TTG ATC
L A W Y Q Q K P G R A P K V L I>

      150     160     170     180     190
TAT AAG GCA TCT ACT TTA GAA AGT GGG GTC CCA TCA AGG TTC AGC GGC
Y K A S T L E S G V P S R F S G>

      200     210     220     230     240
AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGT CTG CAA CCT
S G S G T D F T L T I S S L Q P>

      250     260     270     280
GAA GAT TTT GCA ACT TAC TAC TGT CAA CAG AGT TAC AGT ACC CCG TGG
E D F A T Y Y C Q Q S Y S T P W>

      290     300     310     320
ACG TTC GGC CAA GGG ACC AAG CTG GAG ATC AAA CGT
T F G Q G T K L E I K R

```



Figure 1(b)(i)

```

10      20      30      40
GAC ATC GTG ATG ACC CAG TCT CCA GAC TCC CTG GCT GTG TCT CTG GGC
D I V M T Q S P D S L A V S L G>

50      60      70      80      90
GAG AGG GCC ACC ATC AAC TGC AAG TCC AGC CAG AGT CTT TTA TAC AGC
E R A T I N C K S S Q S L L Y S>

100     110     120     130     140
TAC AAC AAG ATG AAC TAC TTA GCT TGG TAC CAG CAG AAA CCA GGA CAG
Y N K M N Y L A W Y Q Q K P G Q>

150     160     170     180     190
CCT CCT AAG CTG CTC ATT AAC TGG GCA TCT ACC CGG GAA TCC GGG GTC
P P K L L I N W A S T R E S G V>

200     210     220     230     240
CCT GAC CGA TTC AGT GGC AGC GGG TCT GGG ACA GAT TTC ACT CTC ACC
P D R F S G S G S G T D F T L T>

250     260     270     280
ATC AGC AGC CTG CAG GCT GAA GAT GTG GCA GTT TAT TAC TGT CAG CAA
I S S L Q A E D V A V Y C Q Q>

290     300     310     320     330
TAT TAT GCA ACT CCT CTG ACG TTC GGC CAC GGG ACC AAG GTG GAA ATC
Y Y A T P L T F G H G T K V E I>

340
AAA CGT
K R

```

Figure 1(b)(ii)

```

      10      20      30      40
CAC GTT ATA CTG ACT CAG GAC CCT GCT GTG TCT GTG GCC TTG GGA CAG
H V I L T Q D P A V S V A L G Q>

      50      60      70      80      90
ACA GTC AGG ATC ACG TGC CAA GGA GAC AGC CTC AAA AGC TAC TAT GCA
T V R I T C Q G D S L K S Y Y A>

      100     110     120     130     140
AGT TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC ATC TAT
S W Y Q Q K P G Q A P V L V I Y>

      150     160     170     180     190
GGT GAA AAC AGC CGG CCC TCC GGG ATC CCA GAC CGA TTC TCT GGC TCC
G E N S R P S S G I P D R F S G S>

      200     210     220     230     240
AGC TCA GGA AAC ACA GCT TCC TTG ACC ATC ACT GGG GCT CAG GCG GAA
S S G N T A S L T I T G A Q A E>

      250     260     270     280
GAT GAA GCT GAC TAT TAC TGT AAC TCC CGG GAC AGC AGT GGT ACC CAT
D E A D Y Y C N S R D S S G T H>

      290     300     310     320     330
CTA GAA GTG TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT
L E V F G G G T K L T V L G

```

Figure 1(c) (i)

```

      10      20      30      40
CAG GTG CAA CTG GTG GAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AGG
Q V Q L V E S G G G V V Q P G R>

      50      60      70      80      90
TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA CTC ACC TTC AGT AGC TAT
S L R L S C A A S G L T F S S Y>

      100     110     120     130     140
GAC ATG CAC TGG GTC CGC CAG CCT CCA GCC AAG GGG CTG GAG TGG GTG
D M H W V R Q P A K G G L E W V>

      150     160     170     180     190
GCA GTT ATA TCA TAT GAT GGA AGT AGT AAA TAC TAT GCA GAC TCC GTG
A V I S Y D G S S K Y Y A D S V>

      200     210     220     230     240
AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT
K G R F T I S R D N S K N T L Y>

      250     260     270     280
CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT
L Q M N S L R A E D T A V Y C>

      290     300     310     320     330
GCG CGA ACT GGT GAA TAT AGT GGC TAC GAC ACG AGT GGT GTG GAG CTC
A R T G E Y S G Y D T S G V E L>

      340     350     360
TGG GGG CAA GGG ACC ACG GTC ACC GTC TCC TCA
W G Q G T T V T V S S

```

Figure 2(a)(i)

```

      10      20      30      40
GAG GTG CAG CTG GTG GAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AGG
E V Q L V E S G G G V V Q P G R>

      50      60      70      80      90
TCC CTG AGA CTC TCC TGT GCA GCG TCT GGA TTC ACC TTC AGT AGC TAT
S L R L S C A A S G F T F S S Y>

      100      110      120      130      140
GGC ATG CAC TGG GTC CGC CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG
G M H W V R Q A P G K G L E W V>

      150      160      170      180      190
GCA GTT ATA TGG TAT GAT GGA AGT AAT AAA TAC TAT GCA GAC TCC GTG
A V I W Y D G S N K Y Y A D S V>

      200      210      220      230      240
AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT
K G R F T I S R D N S K N T L Y>

      250      260      270      280
CTG CAA ATG GAC AGC CTG AGA GCC GAG GAC ACG GCC GTG TAT TAC TGT
L Q M D S L R A E D T A V Y C>

      290      300      310      320      330
GGA AGA ACG CTG GAG TCT AGT TTG TGG GGC CAA GGC ACC CTG GTC ACC
G R T L E S S L W G Q G T L V T>

      340
GTC TCC TCA
V S S

```

Figure 2(a) (ii)

```

10      20      30      40
GAG ATT CAG CTG GTG GAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AGA
E I Q L V E S G G G V V Q P G R>

50      60      70      80      90
TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT AGC TAT
S L R L S C A A S G F T F S S Y>

100     110     120     130     140
GCT ATG CAC TGG GTC CGC CAG GCT CCA GCC AAG GGG CTG GAG TGG GTG
A M H W V R Q A P A K G L E W V>

150     160     170     180     190
GCA GTT ATA TCA TAT GAT GGA AGC AAT AAA TAC TAC GCA GAC TCC GTG
A V I S Y D G S N K Y Y A D S V>

200     210     220     230     240
AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT
K G R F T I S R D N S K N T L Y>

250     260     270     280
CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCC GTG TAT TAC TGT
L Q M N S L R A E D T A V Y C>

290     300     310     320     330
GCA AGA GCG GGG TTG GAA ACG ACG TGG GGC CAA GGA ACC CTG GTC ACC
A R A G G L E T T W G Q G T L V T>

340     350
GTC TCC TCA AGT GG
V S S S G

```

Figure 2(b) (i)

```

      10      20      30      40
GAT GTT GTG ATG ACT CAG TCT CCA TCC TCC CTG TCT GCA TCT GTA GGA
D   V   V   M   T   Q   S   P   S   S   L   S   A   S   V   G>

      50      60      70      80      90
GAC AGA GTC ACC ATC ACT TGC CGG GCC AGT CAG GGC ATT AGC AAT TAT
D   R   V   T   I   T   C   R   A   S   Q   G   I   S   N   Y>

      100     110     120     130     140
TTA GCC TGG TAT CAG CAA AAA CCA GGG AAA GCC CCT AAG CTC CTG ATC
L   A   W   Y   Q   Q   K   P   G   K   A   P   K   L   L   I>

      150     160     170     180     190
TAT AAG GCA TCT ACT TTA GAA AGT GGG GTC CCA TCA AGG TTC AGT GGC
Y   K   A   S   T   L   E   S   G   V   P   S   R   F   S   G>

      200     210     220     230     240
AGT GGA TCT GGG ACA GAA TTC ACT CTC ACA ATC AGC AGT CTG CAA CCT
S   G   S   G   G   T   E   F   T   L   T   I   S   S   L   Q   P>

      250     260     270     280
GAA GAT TTT GCA ACT TAC TAC TGT CAA CAG AGT TAC AGT ACC CCT CGA
E   D   F   A   T   Y   Y   C   Q   Q   S   Y   S   T   P   R>

      290     300     310     320     330
ACG TTC GGC CAA GGG ACC AAA GTG GAT ATC AAA CGT
T   F   G   G   Q   G   T   K   V   D   I   K   R

```

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Figure 2(b) (ii)

```

      10      20      30      40
TCG TCT GAG CTG ACT CAG GAC CCT GCT GTG TCT GTG GCC TTG GGA CAG
S S E L T Q D P A V S V A L G Q>

      50      60      70      80      90
ACA GTC AGG ATC ACA TGC CAA GGA GAC AGC CTC AGA AGC TAT TAT GCA
T V R I T C Q G D S L R S Y Y A>

      100      110      120      130      140
AGC TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC ATC TAT
S W Y Q Q K P G Q A P V L V I Y>

      150      160      170      180      190
GGT AAA AAC AAC CGG CCC TCA GGG ATC CCA GAC CGA TTC GCT GGC TCC
G K N N R P S G I P D R F A G S>

      200      210      220      230      240
AAC TCA GGA AAC ACA GCT TCC TTG ACC ATC ACT GGG GCT CAG GCG GAG
N S G N T A S L T I T G A Q A E>

      250      260      270      280
GAT GAG GCT GAC TAT TAC TGT AGC TCC CGG GAC AGC AGT GGT AAC CAT
D E A D Y Y C S S R D S S G N H>

      290      300      310      320
GTG GTT TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT
V V F G G G T K L T V L G>

```

Figure 2(b) (iii)

```

10      20      30      40
TCG TCT GAG CTG ACT CAG GAC CCT GCT GTG TCT GTG GCC TTG GGA CAG
S   S   E   L   T   Q   D   P   A   V   S   V   A   L   G   Q>

50      60      70      80      90
ACA GTC AGG ATC ACA TGC CAA GGA GAC AGC CTC AGA AGC TAT TAT GCA
T   V   R   I   T   C   Q   G   D   S   L   R   S   Y   Y   A>

100     110     120     130     140
AGC TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC ATC TAT
S   W   Y   Q   Q   Q   K   P   G   Q   A   P   V   L   V   I   Y>

150     160     170     180     190
GGT AAA AAC AAC CGG CCC TCA GGG ATC CCA GAC CGA TTC TCT GGC TCC
G   K   N   N   R   P   S   G   I   P   D   R   F   S   G   S>

200     210     220     230     240
AGC TCA GGA AAC ACA GCT TCC TTG ACC ATC ACT GGG GCT CAG GCG GAA
S   S   G   N   T   A   S   L   T   I   T   G   A   Q   A   E>

250     260     270     280
GAT GAG GCT GAC TAT TAC TGT AAC TCC CGG GAC AGC AGT AGT ACC CAT
D   E   A   D   Y   Y   C   N   S   R   D   S   S   S   T   H>

290     300     310     320     330
CGA GGG GTG TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT
R   G   G   V   F   G   G   G   T   K   L   T   V   L   G

```



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Figure 2(b) (iv)

```

10      20      30      40
GAA GTT GTG CTG ACT CAG TCT CCA TCC TCC CTG TCT GCA TCT GTA GGA
E V V L L T Q S P S S L S A S V G>

50      60      70      80      90
GAC AGA GTC ACC ATC ACT TGC CGG GCA AGT CAG GGC ATT GGA GAT GAT
D R V T I T C R A S Q G I G D D>

100     110     120     130     140
TTG GGC TGG TAT CAG CAG AAG CCA GGG AAA GCC CCT ATC CTC CTG ATC
L G W Y Q Q K P G K A P I L L I>

150     160     170     180     190
TAT GGT ACA TCC ACT TTA CAA AGT GGG GTC CCG TCA AGG TTC AGC GGC
Y G T S T L Q S G V P S R F S G>

200     210     220     230     240
AGT GGA TCT GGC ACA GAT TTC ACT CTC ACC ATC AAC AGC CTG CAG CCT
S G S G T D F T L T I N S L Q P>

250     260     270     280
GAA GAT TTT GCA ACT TAT TAC TGT CTA CAA GAT TCC AAT TAC CCG CTC
E D F A T Y Y C L Q D S N Y P L>

290     300     310     320
ACT TTC GGC GGA GGG ACA CGA CTG GAG ATT AAA CGT
T F G G G T R L E I K R

```

Figure 2(b) (v)

```

10      20      30      40
TCG TCT GAG CTG ACT CAG GAC CCG GCT GTG TCT GTG GCC TTG GGA CAG
S   S   E   L   T   Q   D   P   A   V   S   V   A   L   G   Q>

50      60      70      80      90
ACA GTC AGG ATC ACA TGC CAA GGA GAC AGC CTC AGA AAC TAT TAT GCA
T   V   R   I   T   C   Q   G   D   S   L   R   N   Y   Y   A>

100     110     120     130     140
AAC TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC ATC TAT
N   W   Y   Q   Q   Q   K   P   G   Q   A   P   V   L   V   I   Y>

150     160     170     180     190
GGT AAA AAC AAC CGG CCC TCA GGG ATC CCA GAC CGA TTC TCT GGC TCC
G   K   N   N   R   P   S   G   I   P   D   R   F   S   G   S>

200     210     220     230     240
AGC TCA GGG AAC ACA GCT TCC TTG ACC ATC ACT GGG GCT CGG GCG GAA
S   S   G   N   T   A   S   L   T   I   T   G   A   R   A   E>

250     260     270     280
GAT GAG GGT GTC TAT TAC TGT AAC TCC CGG GAC AGC AGT GGT GCG GTT
D   E   G   V   Y   Y   C   N   S   R   D   S   S   G   A   V>

290     300     310     320
TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT
F   G   G   G   T   K   L   T   V   L   G

```

|               |                                   |
|---------------|-----------------------------------|
| PARENT (1-B2) | A R T G E Y S G Y D S S G V D V W |
| 27-C1         | A R T G E Y S G Y D T S G V E L W |
| 27-D7         | A R T R E Y S G H D S S G V D D W |
| 27-E10        | A R T G P F S G Y D S S G E D V R |
| 27-H1         | A R T E E Y S G Y D S S G V D V W |
| 27-E2         | A Q T R E Y T G Y D S S G V D V W |
| 28-A11        | A R T E E Y S G F D S T G E D V W |
| 28-E12        | A R T E E F S G Y D S S G V D V W |
| 28-H10        | A R T G E Y S G Y H S S G V D V R |
| 31-G2         | A R T E E F S G Y D S S G V D V W |
| 30-B6         | A R A G P F S G Y D S S G E D V R |
| 30-E9         | A R T G P F S G Y D S S G E D V W |
| 30-F6         | A R T E E F S G Y D S S G V D V W |
| 30-D2         | A R T G E Y S G Y D S S G E L V W |
| 31-A2         | A R T E E F S G Y D S T G E E V W |
| 31-E11        | A R T E E F S G Y D S S G V D V W |
| 31-F1         | A R T G E Y S G Y D S S G E D V W |

Figure 4

```

10      20      30      40
TCG TCT GAG CTG ACT CAG GAC CCT GCT GTG TCT GTG GCC TTG GGA CAG
S S E L T Q D P A V S V A L G Q>

50      60      70      80      90
ACA GTT AGG ATC ACT TCC CAA GGA GAC AGT CTC AGA AGC TAT TAC ACA
T V R I T S Q G D S L R S Y Y T>

100     110     120     130     140
AAC TGG TTT CAG CAG AAG CCA GGA CAG CCC CCT CTA CTT GTC GTC TAT
N W F Q Q Q K P G Q P L L V V Y>

150     160     170     180     190
GCT AAA AAT AAG CGG CCC TCA GGG ATC CCA GAC CGA TTC TCT GGC TCC
A K N K R P S G I P D R F S S>

200     210     220     230     240
AGC TCA GGA AAC ACA GCT TCC TTG ACC ATC ACT GGG GCT CAG GCG GAA
S S G N T A S L T I T G A Q A E>

250     260     270     280
GAT GAG GCT GAC TAT TAC TGT CAT TCC CGG GAC AGC AGT CAT AAC CAT
D E A D Y Y C H S R D S S G H>

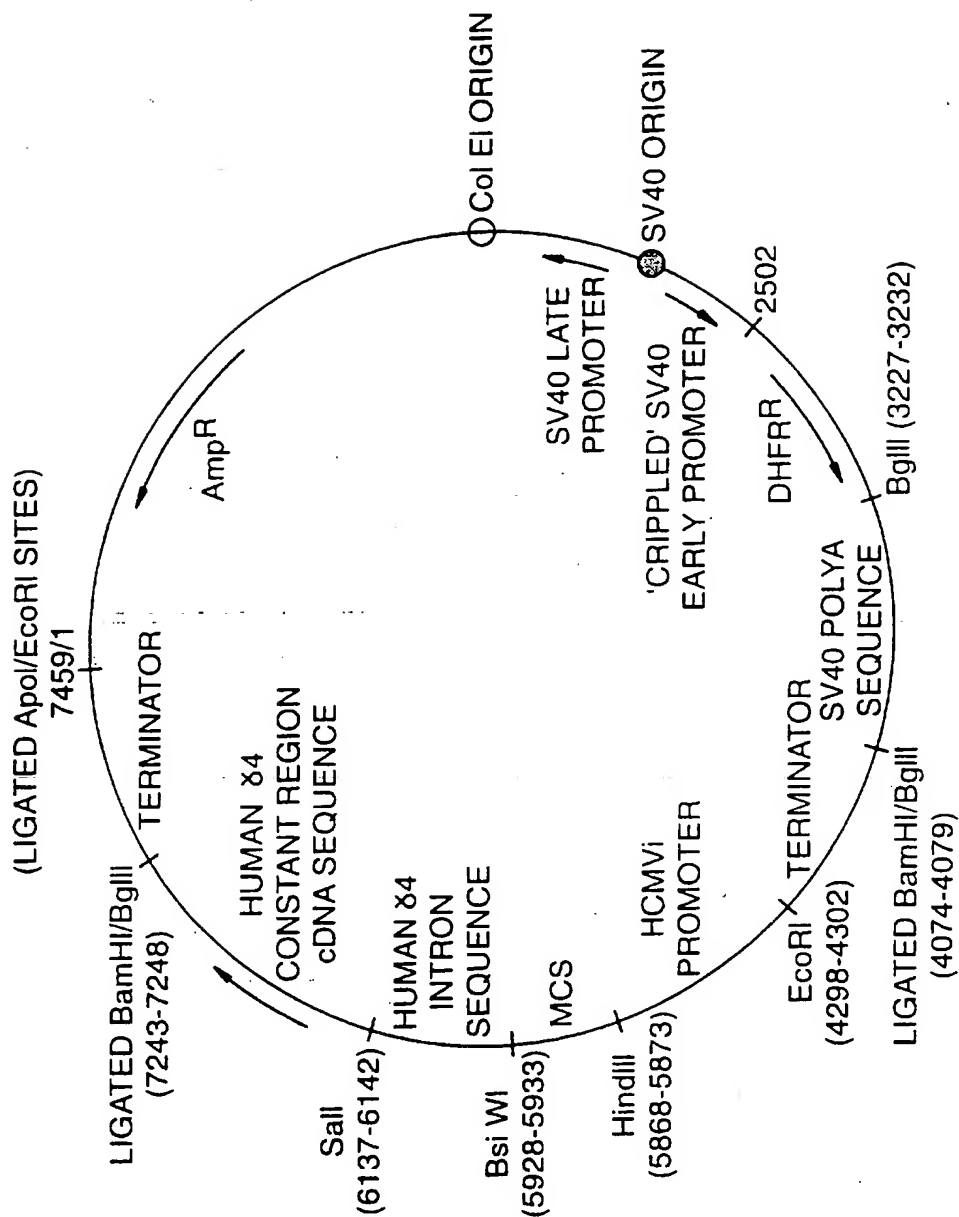
290     300     310     320
GTG CTT TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT
V L F G G G T K L T V L G

```

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H  
i  
n  
d  
I  
I  
I  
I  
aagcttgcgcgccaccatggactggacctggcgcgtgttttgectgctcgccgtggccct  
1 -----+-----+-----+-----+ 60  
ctcgaacggcggtgttacctgacctggaccgcgcacaaaacggacgagcggcaccgggga  
a K L A A T H D W T W R V F C L L A V A P -  
  
S P B  
f s s  
i t E  
I I I  
ggggccacagccaggtgcaactgcagcagtcctgggtgccaaagggaccacggtcaccgtct  
61 -----+-----+-----+-----+ 120  
ccccgggtgtcgggtccacgttgacgtcgtcaggccacggttccctgggtgccagtggcaga  
a G A H S Q V Q L Q Q S G A K G P R S P S -  
  
B E  
a c  
m o  
H R  
I I  
cctcaggtgagtggtatccgaattc  
121 -----+-----+-----+ 144  
ggagtccactcacctaggcttaag  
a P Q V S G S E F -

Fig.6.



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H

i

n

d

I

I

I

I

1 aagcttcgccaccatgggatggagctgtatcctctcttcttggtagcaacagctacagg 60  
-----+-----+-----+-----+-----+  
1 ttcgaaagcgggtggtaccctacctcgacatagtaggagaagaaccatcgttgcgatgtcc

M G W S C I I L F L V A T A T

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61 attccccgagtgctcatcgtccgaactccagacctgtatatataccccactgttactgtagg

A

P

a

L

I

S

a

c

I

121 actttgcctttctctccacaggtgtgcactccgacattgagctcaccagctctccagaca 180  
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121 tgaaacggaaagagaggtgtccacacgtgaggctgtaactcgagtgaggtcagaggtctgt

G V H S D I E L

X

h

o

I

B

a

m

H

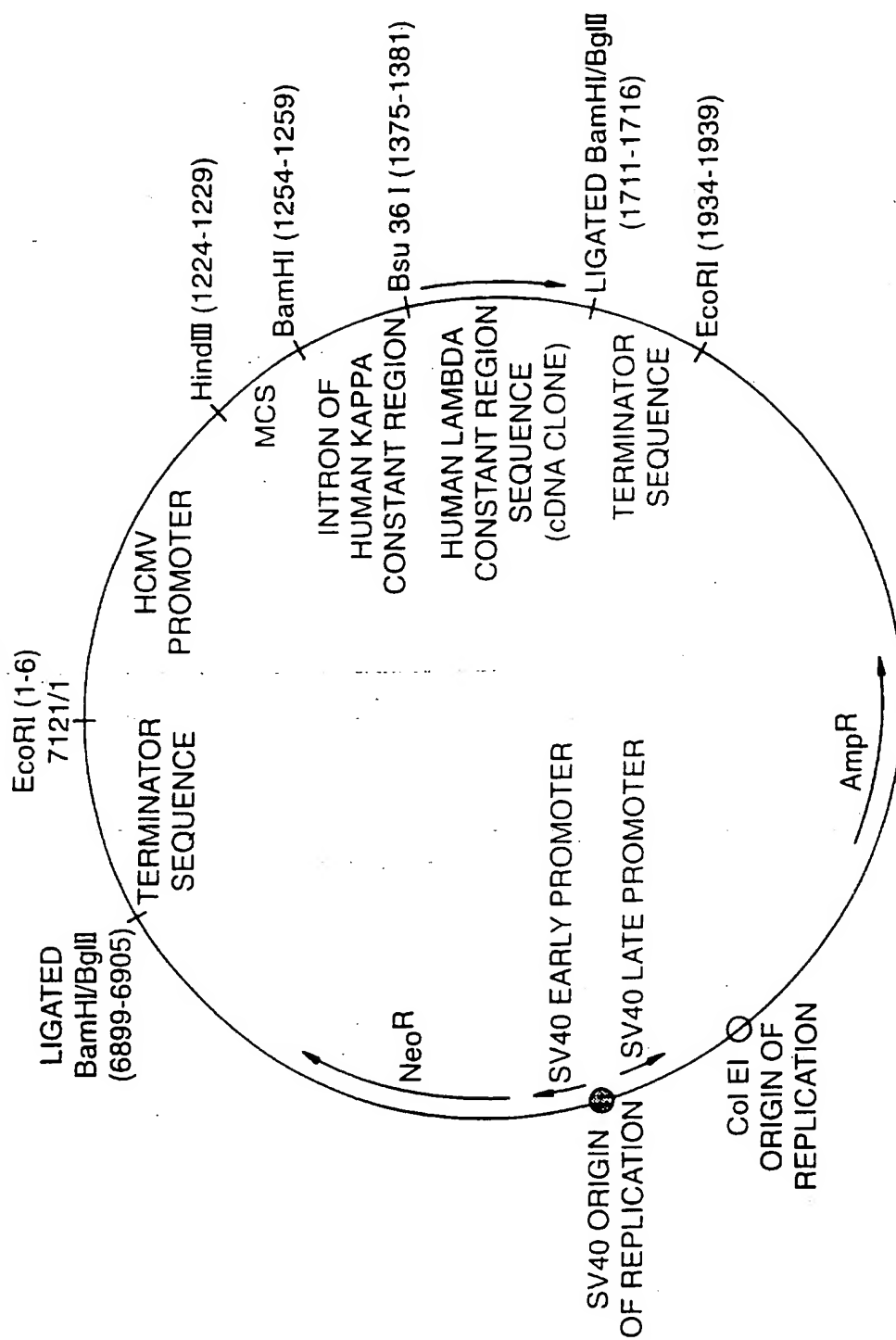
I

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L E L K

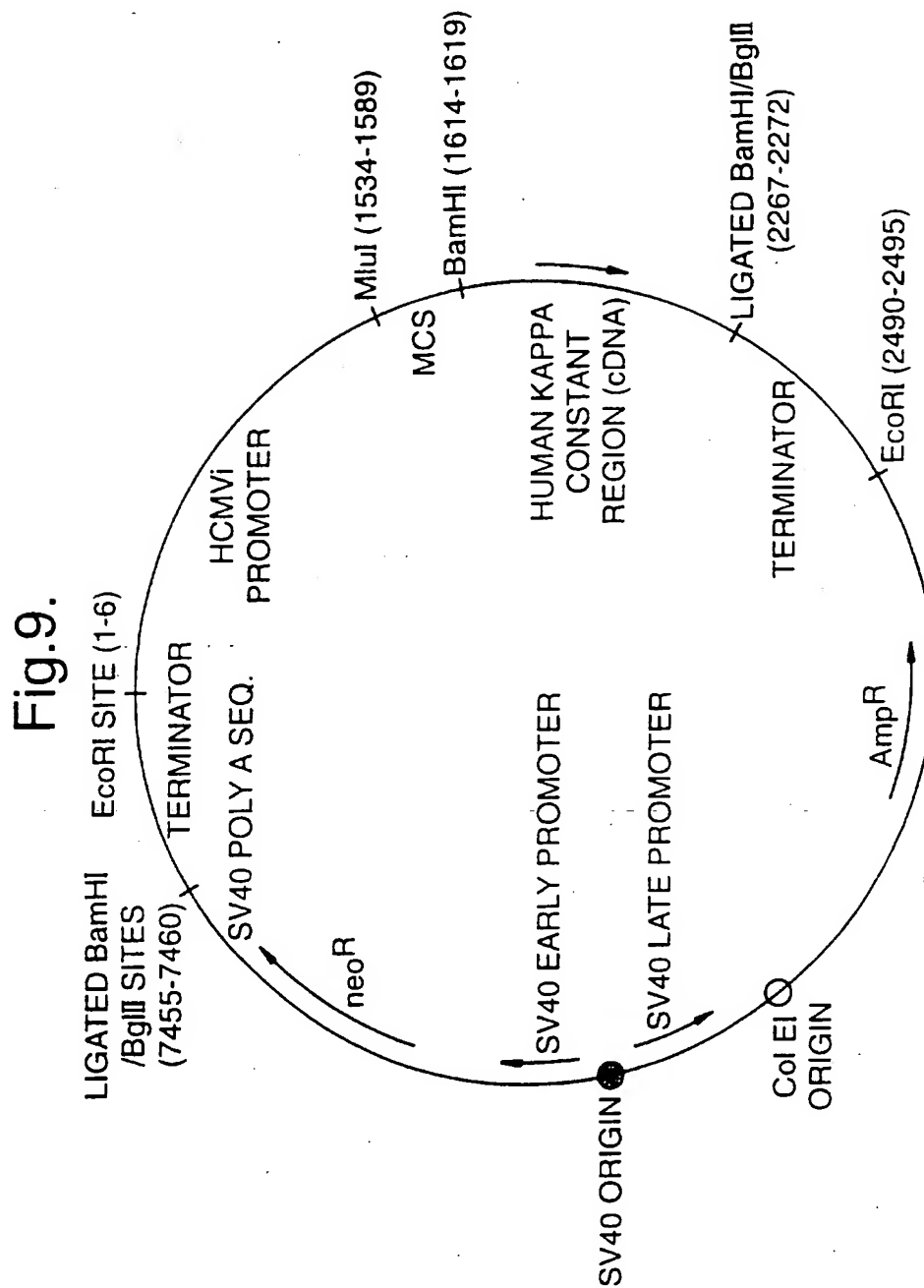
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Fig.8.

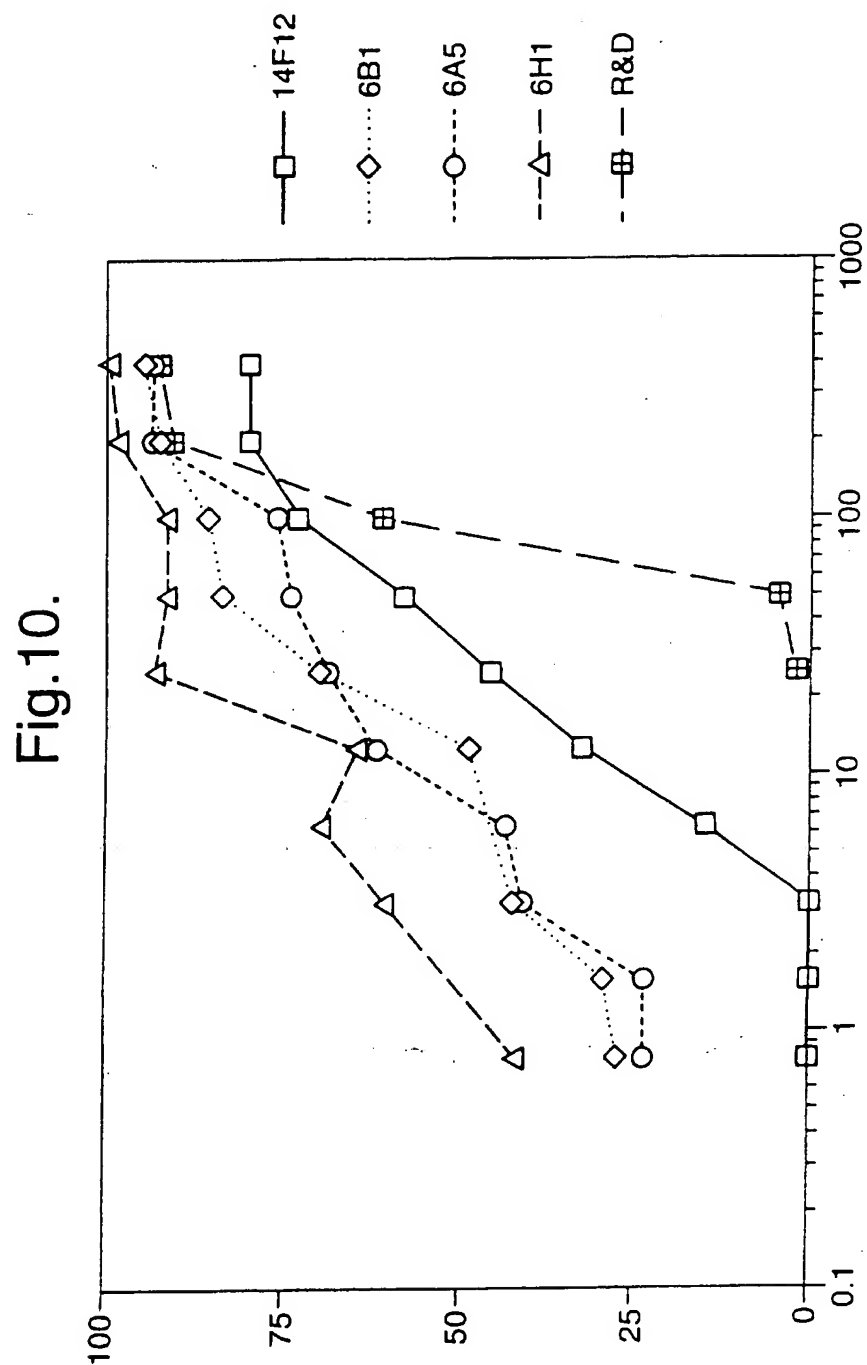




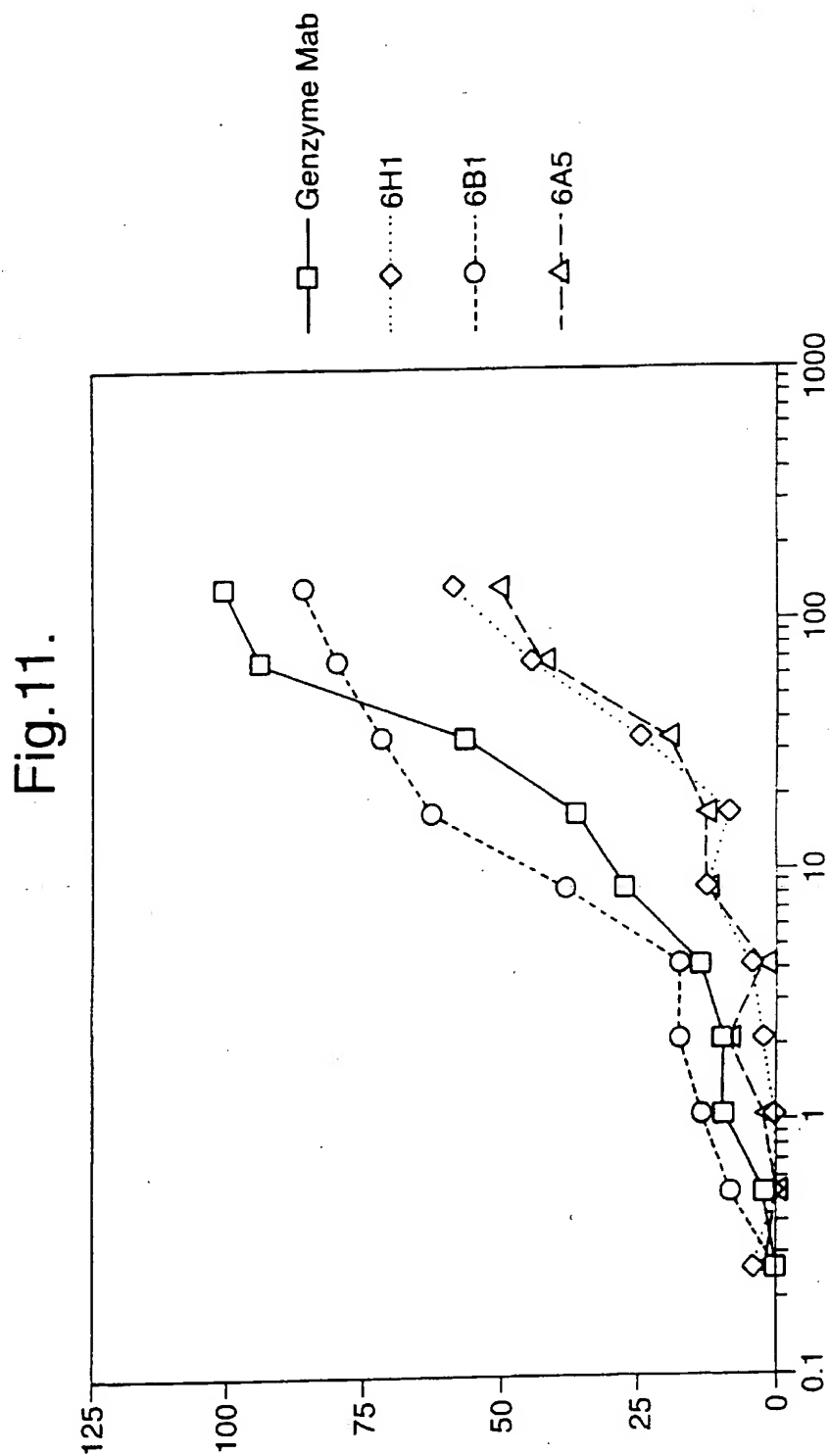
20/38



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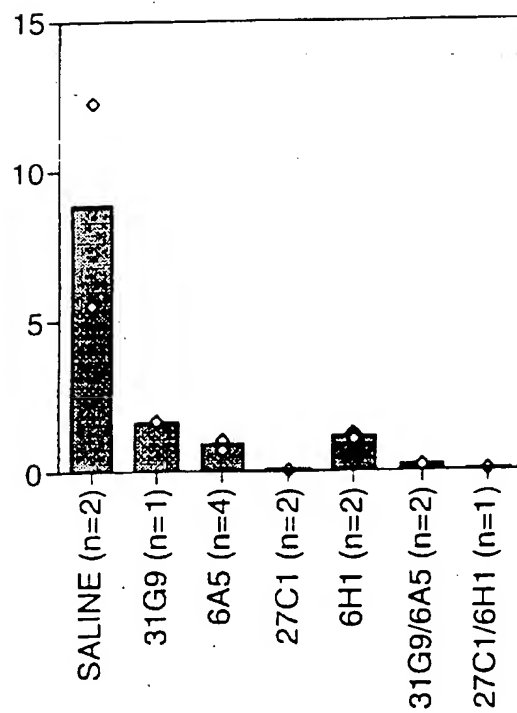
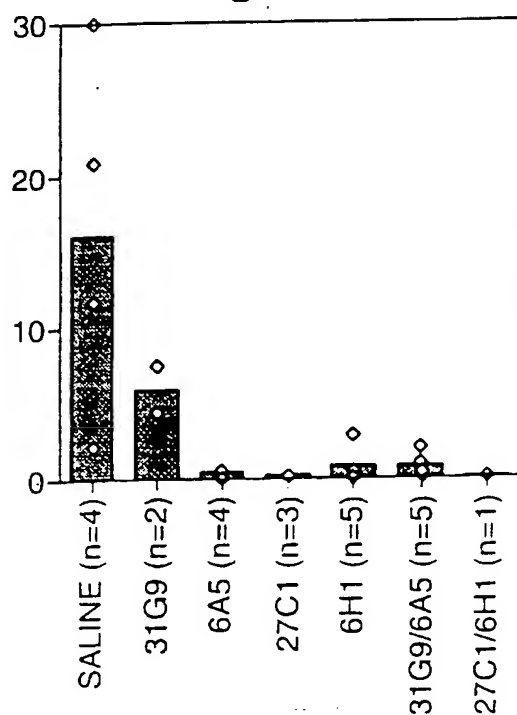


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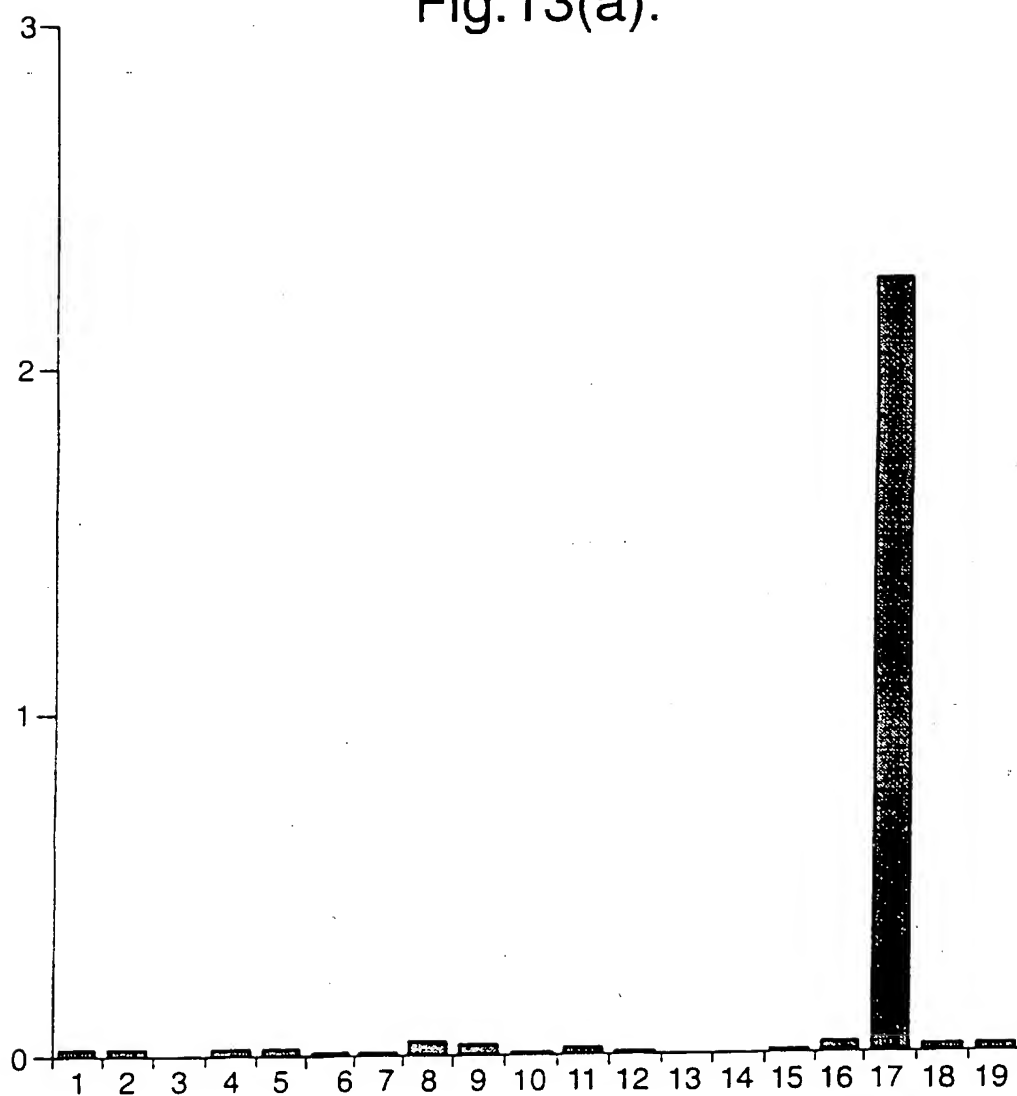
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Fig.12.



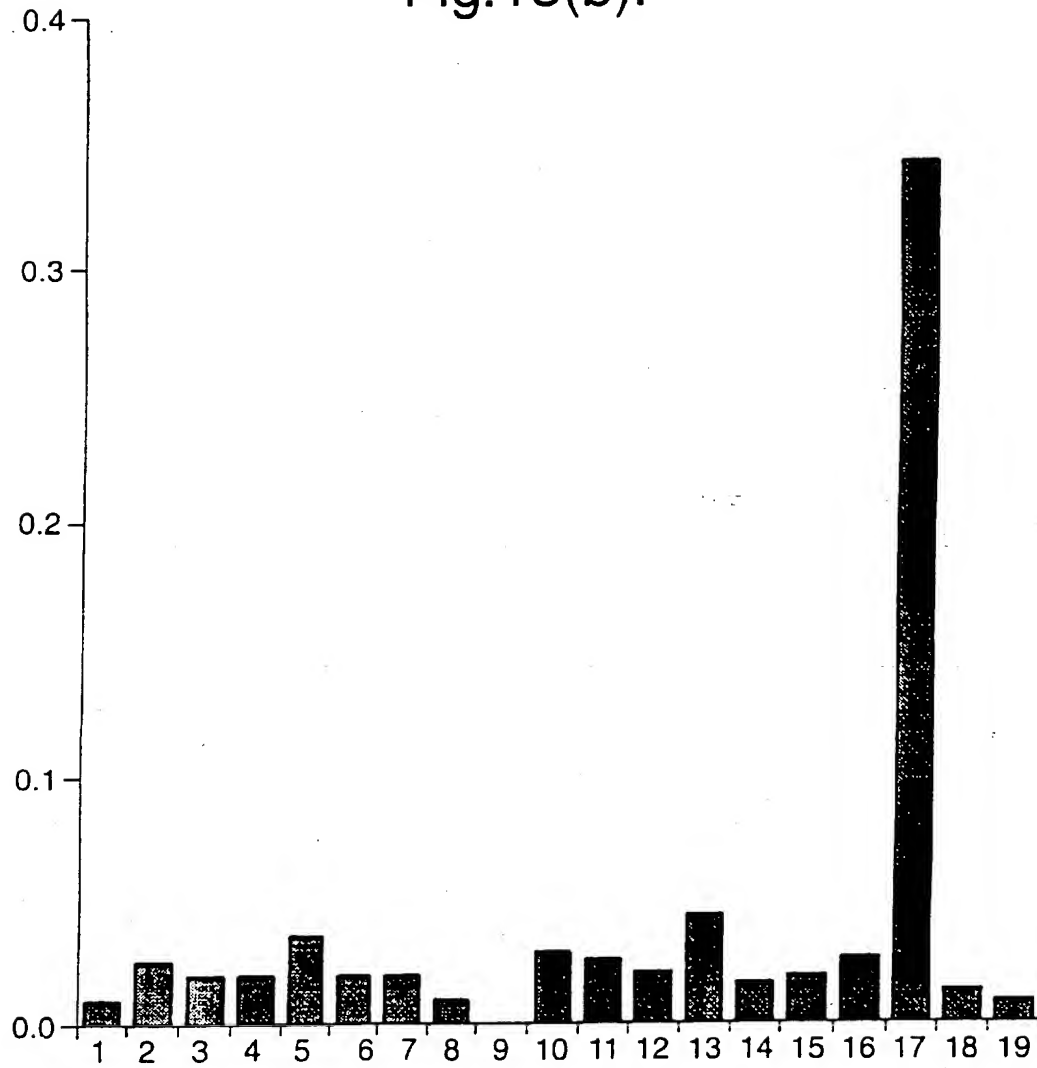
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Fig.13(a).



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Fig.13(b).



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Figure 14

```

10  20  30  40
GAA ATT GTG CTG ACT CAG TCT CCA TCC TCC CTG TCT GCA TCT GTA GGA
E  I  V  L  T  Q  S  P  S  S  L  S  A  S  V  G>

50  60  70  80  90
GAC AGA GTC ACC ATC ACT TGC CGG GCA AGT CAG GGC ATT GGA GAT GAT
D  R  V  T  I  T  C  R  A  S  Q  G  I  G  D  D>

100 110 120 130 140
TTG GGC TGG TAT CAG CAG AAG CCA GGG AAA GCC CCT ATC CTC CTG ATC
L  G  W  Y  Q  Q  K  P  G  K  A  P  I  L  L  I>

150 160 170 180 190
TAT GGT ACA TCC ACT TTA CAA AGT GGG GTC CCG TCA AGG TTC AGC GGC
Y  G  T  S  T  L  Q  S  G  V  P  S  R  F  S  G>

200 210 220 230 240
AGT GGA TCT GGC ACA GAT TTC ACT CTC ACC ATC AAC AGC CTG CAG CCT
S  G  S  G  T  D  F  T  L  T  I  N  S  L  Q  P>

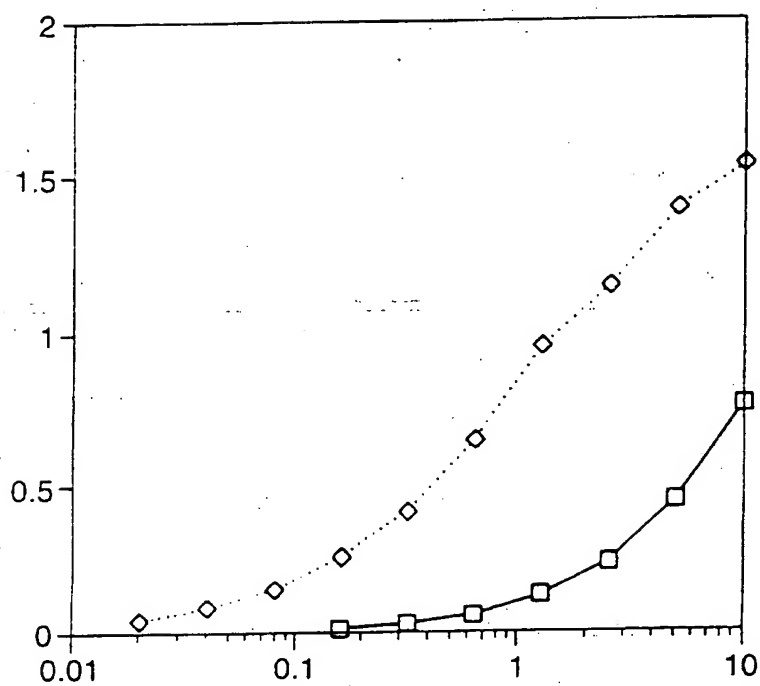
250 260 270 280
GAA GAT TTT GCA ACT TAT TAC TGT CTA CAA GAT TCC AAT TAC CCG CTC
E  D  F  A  T  Y  Y  C  L  Q  D  S  N  Y  P  L>

290 300 310 320
ACT TTC GGC GGA GGG ACA CGA CTG GAG ATT AAA CGT
T  F  G  G  G  T  R  L  E  I  K  R>

```

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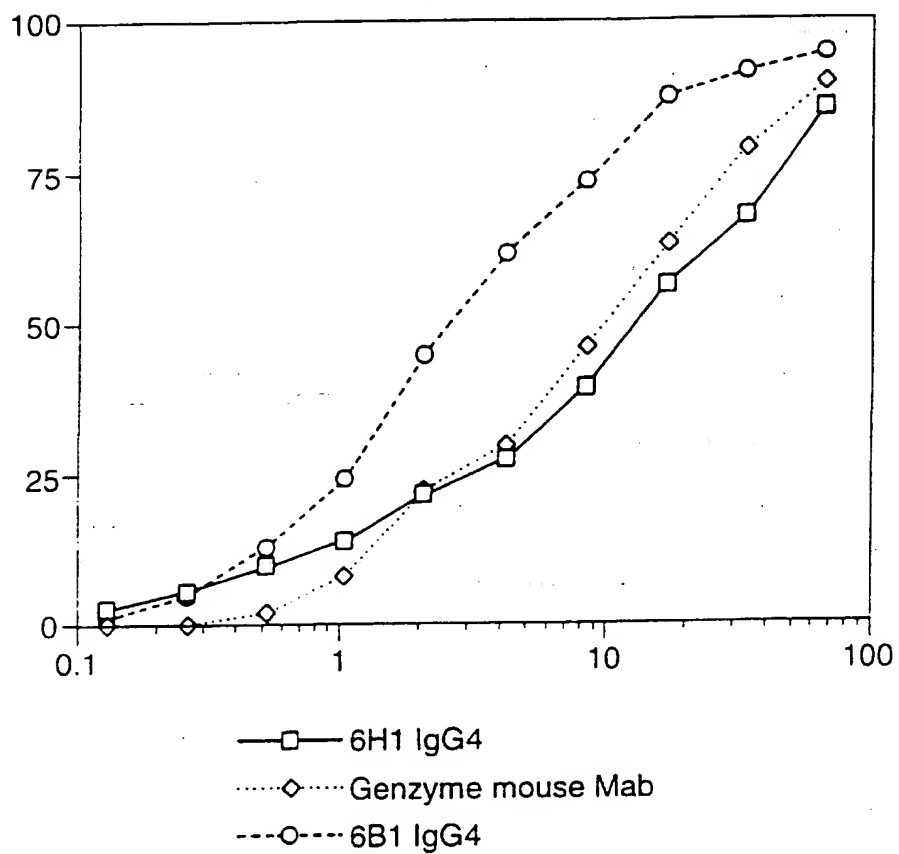
Fig.15.





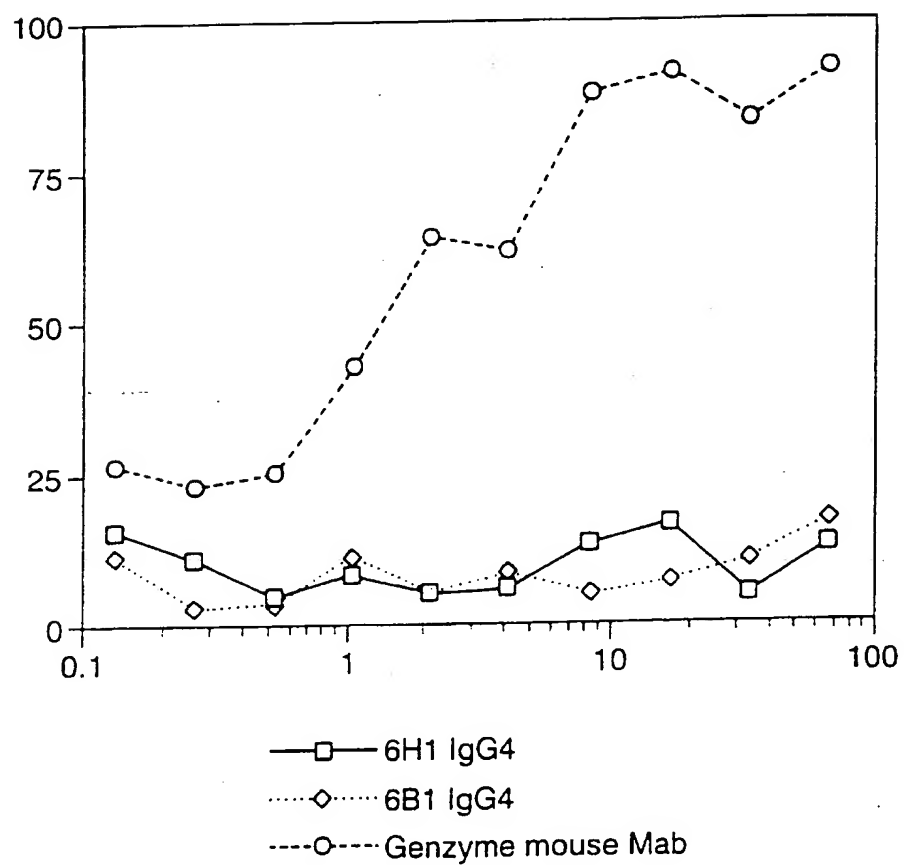
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Fig.16.



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Fig.17.



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Fig.18.

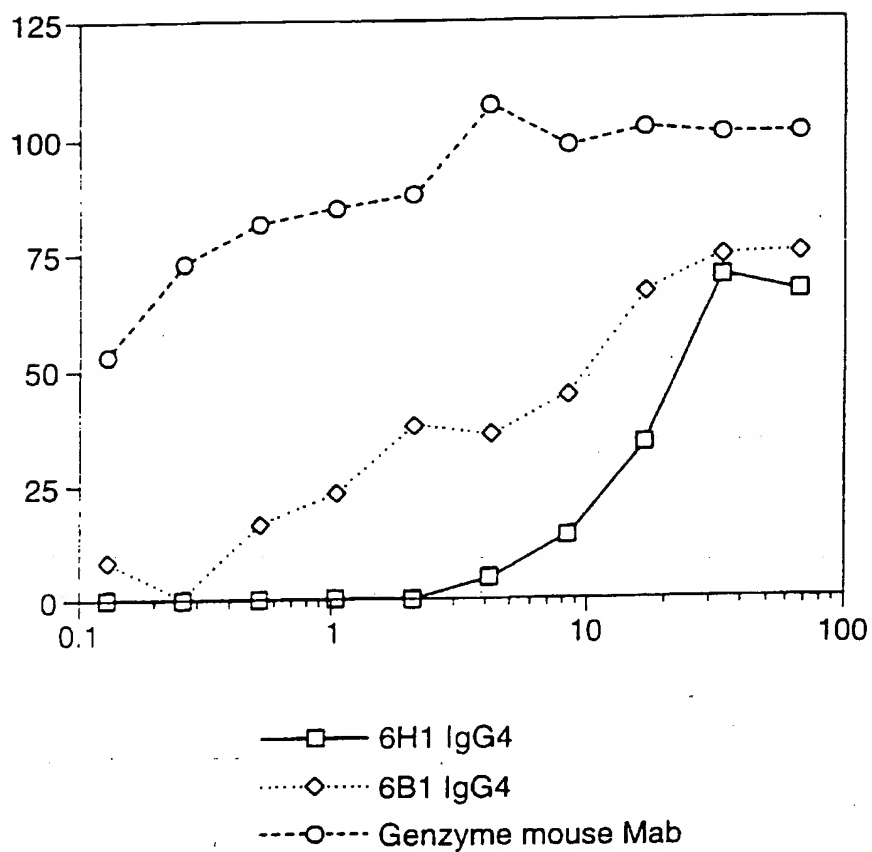


Figure 19

(i)

```

10 20 30 40
GAG GTG CAG CTG GTG GAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AGG
E V Q L V E S G G G V V Q P G R>

50 60 70 80 90
TCC CTG AGA CTC TCC TGT GCA GCG TCT GGA TTC ACC TTC AGT AGC TAT
S L R L S C A A S G F T F S S Y>

100 110 120 130 140
GGC ATG CAC TGG GTC CGC CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG
G M H W V R Q A P G G K G L E W V>

150 160 170 180 190
GCA GTT ATA TGG TAT GAT GGA AGT AAT AAA TAC TAT GCA GAC TCC GTG
A V I W Y D G S N K Y Y A D S V>

200 210 220 230 240
AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT
K G R F T I S R D N S K H T L Y>

250 260 270 280
CTG CAA ATG GAC AGC CTG AGA GCC GAG GAC ACG GCC GTG TAT TAC TGT
L Q M D S L R A E D T A V Y C>

290 300 310 320 330
GGA AGA ACG CTG GAG TCT AGT TTG TGG GGC CAA GGC ACC CTG GTC ACC
G R T L E S S L W G Q G T L V T>

340
GTC TCC TCA
V S S

```

Figure 19 (ii)

```

10      20      30      40
TCG TCT GAG CTG ACT CAG GAC CCT GCT GTG TCT GTG GCC TTG GGA CAG
S   S   E   L   T   Q   D   P   A   V   S   V   A   L   G   Q>

50      60      70      80      90
ACA GTC AGG ATC ACA TGC CAA GGA GAC AGC CTC AGA AGC TAT TAT GCA
T   V   R   I   T   C   Q   G   D   S   L   R   S   Y   Y   A>

100     110     120     130     140
AGC TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC ATC TAT
S   W   Y   Q   Q   K   P   G   Q   A   P   V   L   V   I   Y>

150     160     170     180     190
GGT AAA AAC AAC CGG CCC TCA GGG ATC CCA GAC CGA TTC TCT GGC TCC
G   K   N   N   R   P   S   G   I   P   D   R   F   S   G   S>

200     210     220     230     240
AGC TCA GGA AAC ACA GCT TCC TTG ACC ATC ACT GGG GCT CAG GCG GAA
S   S   G   N   T   A   S   L   T   I   T   G   A   Q   A   E>

250     260     270     280
GAT GAG GCT GAC TAT TAC TGT AAC TCC CGG GAC AGC AGT AGT ACC CAT
D   E   A   D   Y   Y   C   N   S   R   D   S   S   S   T   H>

290     300     310     320     330
CGA GGG GTG TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT
R   G   V   F   G   G   G   G   T   K   L   T   V   L   G

```

Figure 19 (iii)

```

10      20      30      40
TCG TCT GAG CTG ACT CAG GAC CCT GCT GTG TCT GTG GCC TTG GGA CAG
S   S   E   L   T   Q   D   P   A   V   S   V   S   V   A   L   G   Q>

50      60      70      80      90
ACA GTC AGG ATC ACA TGC CAA GGA GAC AGC CTC AGA AGC TAT TAT GCA
T   V   R   I   T   C   Q   G   D   S   L   R   S   Y   Y   A>

100     110     120     130     140
AGC TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC ATC TAT
S   W   Y   Q   Q   Q   K   P   G   G   Q   A   P   V   L   V   I   Y>

150     160     170     180     190
GGT AAA AAC AAC CGG CCC TCA GGG ATC CCA GAC CGA TTC GCT GGC TCC
G   K   N   N   R   P   S   G   I   P   D   R   F   A   G   S>

200     210     220     230     240
AAC TCA GGA AAC ACA GCT TCC TTG ACC ATC ACT GGG GCT CAG GCG GAG
N   S   G   N   T   A   S   L   T   I   T   G   A   Q   A   E>

250     260     270     280
GAT GAG GCT GAC TAT TAC TGT AGC TCC CGG GAC AGC AGT GGT AAC CAT
D   E   A   D   Y   Y   C   S   S   R   D   S   S   G   N   H>

290     300     310     320
GTG GTT TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT
V   V   F   G   G   G   G   T   K   L   T   V   L   G

```

Figure 19(iv)

```

      10      20      30      40
GAT GTT GTG ATG ACT CAG TCT CCA TCC CTG TCT GCA TCT GTA GGA
D V V M T Q S P S S L S A S V G>

      50      60      70      80      90
GAC AGA GTC ACC ATC ACT TGC CGG GCC AGT CAG GGC ATT AGC AAT TAT
D R V T I T C R A S Q G I S N Y>

      100     110     120     130     140
TTA GCC TGG TAT CAG CAA AAA CCA GGG AAA GCC CCT AAG CTC CTG ATC
L A W Y Q Q K P G K A P K L L I>

      150     160     170     180     190
TAT AAG GCA TCT ACT TTA GAA AGT GGG GTC CCA TCA AGG TTC AGT GGC
Y K A S T L E S G V P S R F S G>

      200     210     220     230     240
AGT GGA TCT GGG ACA GAA TTC ACT CTC ACA ATC AGC AGT CTG CAA CCT
S G S G T E F T L T I S S L Q P>

      250     260     270     280
GAA GAT TTT GCA ACT TAC TAC TGT CAA CAG AGT TAC AGT ACC CCT CGA
E D F A T Y Y C Q Q Q S Y S T P R>

      290     300     310     320
ACG TTC GGC CAA GGG ACC AAA GTG GAT ATC AAA CGT
T F G Q G T K V D I K R

```

Fig.20.

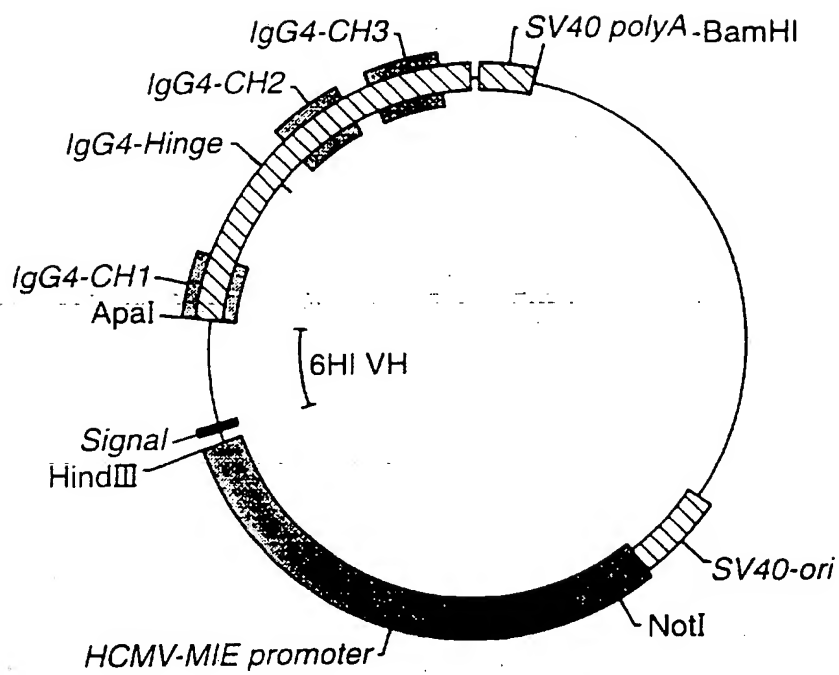
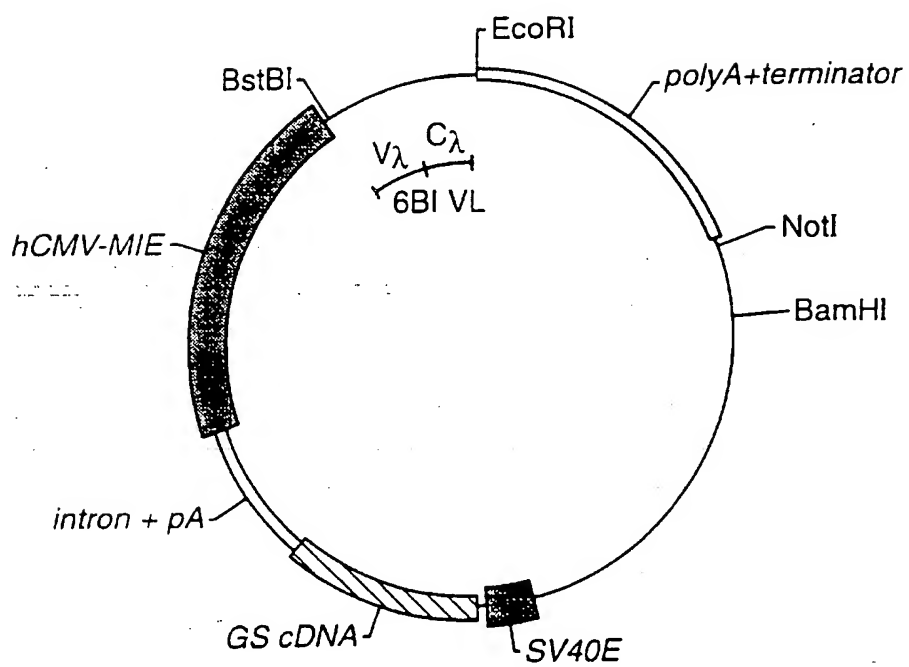




Fig.21.



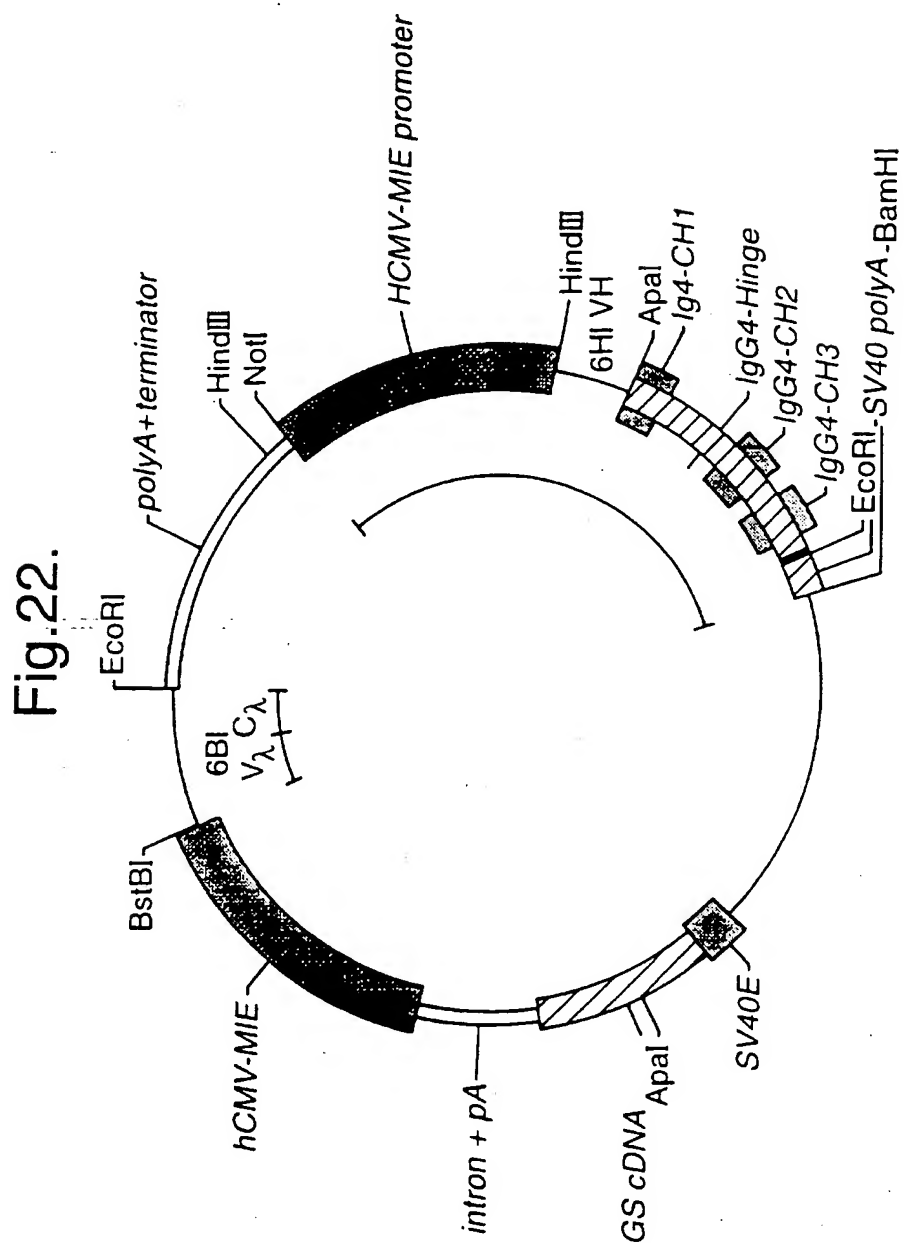
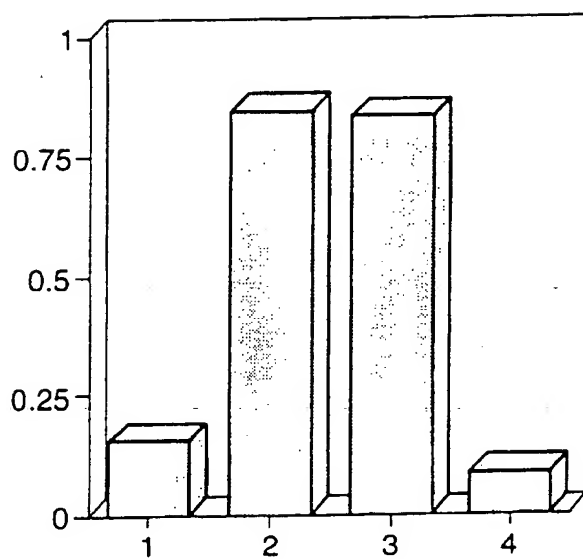


Fig.23.



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 96/02450

|  |   |   |
|--|---|---|
| <b>A. CLASSIFICATION OF SUBJECT MATTER</b><br>IPC 6 C12N5/10 C12N15/13 C07K16/22 A61K39/395  |   |   |
| According to International Patent Classification (IPC) or to both national classification and IPC  |   |   |
| <b>B. FIELDS SEARCHED</b><br>Minimum documentation searched (classification system followed by classification symbols)<br>IPC 6 C12N C07K A61K   |   |   |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  |   |   |
| Electronic data base consulted during the international search (name of data base and, where practical, search terms used)   |   |   |
| <b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>  |   |   |
| Category *   | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No.                       |
| X  | WO 93 21945 A (UNIV CALIFORNIA ;JOLLA<br>CANCER RES FOUND (US)) 11 November 1993<br><br>see abstract and page 6 line6 -line 23<br>--- | 1,2,<br>31-33,<br>35-41,<br>43,<br>54-57,59 |
| X  | WO 92 17206 A (UNIV MANCHESTER) 15 October<br>1992<br>cited in the application<br><br>see claims<br>---<br>-/--                       | 1-3,<br>31-33,<br>35-41,<br>54-57           |
| <div style="display: flex; justify-content: space-between;"> <span><input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.</span> <span><input checked="" type="checkbox"/> Patent family members are listed in annex.</span> </div>  |   |   |
| <div style="display: flex;"> <div style="flex: 1;"> <p>* Special categories of cited documents :</p> <p>* "A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>* "E" earlier document but published on or after the international filing date</p> <p>* "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>* "O" document referring to an oral disclosure, use, exhibition or other means</p> <p>* "P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>* "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>* "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>* "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>* "&amp;" document member of the same patent family</p> </div> </div> |   |   |
| Date of the actual completion of the international search  | Date of mailing of the international search report  |   |
| 17 February 1997   | 26.02.97  |   |
| Name and mailing address of the ISA<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+ 31-70) 340-3016   |   | Authorized officer<br><br>Müller, F         |

# INTERNATIONAL SEARCH REPORT

Inter. App. No.

PCT/GB 96/02450

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No.                             |
|------------|--|---|
| X          | <p>WO 91 04748 A (JOLLA CANCER RES FOUND) 18 April 1991<br/>cited in the application</p> <p>see claims 12,5-7,13-21 and page 11, line 26 - page 12, line 13, examples III, VII<br/>---</p>   | <p>1-3,<br/>31-33,<br/>35-43,<br/>54-59</p>       |
| X          | <p>NATURE,<br/>- 26 July 1990<br/>pages 371-374, XP002025313<br/>BORDER W. A. ET AL., : "Suppression of experimental glomerulonephritis by antiserum against transforming growth factor betal"<br/>cited in the application<br/>see the whole document<br/>---</p>   | <p>1,2,15,<br/>31-33,<br/>35-43,<br/>54-59</p>    |
| X          | <p>J. IMMUNOLOGY,<br/>vol. 145, - 1 September 1990<br/>pages 1415-1422, XP002025314<br/>LUCAS C. ET AL., : "The autocrine production of transforming growth factor-beta1 during lymphocyte activation"<br/>cited in the application<br/>see summary and p.1420, 2.column, 3.paragraph, table 1<br/>---</p> | <p>1-3,13,<br/>15,27,<br/>31-33,<br/>35,36,38</p> |
| X          | <p>J. IMMUNOLOGY,<br/>vol. 142, no. 5, - 5 March 1989<br/>pages 1536-1541, XP002025315<br/>DASCH J.R. ET AL., : "Monoclonal ,antibodies recognizing transforming growth factor-beta"<br/>cited in the application<br/>see results and discussion<br/>---</p>   | <p>1-3,27,<br/>31-33,<br/>35,36,38</p>            |
| X          | <p>J. CELL SCIENCE,<br/>- March 1995<br/>pages 985-1002, XP002025316<br/>SHAH M. ET AL., : "Neutralisation of TGF-betal and TGF-beta2 or exogenous addition of TGF-beta3 to cutaneous rat wounds reduces scarring"<br/>see the whole document<br/>---</p>  | <p>1-3,13,<br/>15,27</p>                          |
| Y          | <p>see the whole document<br/>---</p>  | <p>30-34,<br/>45-51</p>                           |
| Y          | <p>WO 94 13804 A (CAMBRIDGE ANTIBODY TECH ;MEDICAL RES COUNCIL (GB); HOLLIGER KASPAR) 23 June 1994<br/>see the whole document<br/>---</p>  | <p>30-34,<br/>45-51</p>                           |

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# INTERNATIONAL SEARCH REPORT

Inter nal Application No  
PCT/GB 96/02450

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT |  |   |
|--|--|---|
| Category *   | Citation of document, with indication, where appropriate, of the relevant passages             | Relevant to claim No.                       |
| P,X  | GB 2 288 118 A (UNIV MANCHESTER) 11<br>October 1995<br><br>see the whole document<br>---       | 1-3,13,<br>15,<br>30-33,<br>35-43,<br>54-59 |
| E  | US 5 571 714 A (DASCH JAMES R ET AL) 5<br>November 1996<br><br>see the whole document<br>----- | 1-3,13,<br>15,27,<br>31-33,<br>35,36        |

# INTERNATIONAL SEARCH REPORT

information on patent family members

Int- Application No

PCT/GB 96/02450

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|---|---------------------|----------------------------|---------------------|
| WO-A-9321945                              | 11-11-93            | AU-A- 4111893              | 29-11-93            |
| WO-A-9217206                              | 15-10-92            | AU-B- 661840               | 10-08-95            |
|   |                     | AU-A- 1436892              | 02-11-92            |
|   |                     | CA-A- 2105652              | 29-09-92            |
|   |                     | EP-A- 0585242              | 09-03-94            |
|   |                     | JP-T- 6506205              | 14-07-94            |
| WO-A-9104748                              | 18-04-91            | AU-B- 654938               | 01-12-94            |
|   |                     | AU-A- 6612590              | 28-04-91            |
|   |                     | CA-A- 2065860              | 30-03-91            |
|   |                     | EP-A- 0494264              | 15-07-92            |
|   |                     | JP-B- 7080780              | 30-08-95            |
|   |                     | JP-T- 5503076              | 27-05-93            |
| WO-A-9413804                              | 23-06-94            | AU-A- 5654894              | 04-07-94            |
|   |                     | CA-A- 2150262              | 23-06-94            |
|   |                     | EP-A- 0672142              | 20-09-95            |
|   |                     | JP-T- 8504100              | 07-05-96            |
|   |                     | AU-A- 7621494              | 10-04-95            |
|   |                     | CA-A- 2169620              | 30-03-95            |
|   |                     | EP-A- 0720624              | 10-07-96            |
|   |                     | WO-A- 9508577              | 30-03-95            |
| GB-A-2288118                              | 11-10-95            | AU-A- 2077895              | 17-10-95            |
|   |                     | EP-A- 0754058              | 22-01-97            |
|   |                     | WO-A- 9526203              | 05-10-95            |
| US-A-5571714                              | 05-11-96            | NONE                       |                     |

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